

# Mapping of Secondary Metabolism in Biotechnologically Important aspergillus Species

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Center for Microbial Biotechnology
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# Preface

This PhD thesis is the product of my PhD studies at Center for Microbial Biotechnology at Systems Biology, Technical University of Denmark and financed by "Det Frie Forskningsråd, Teknologi og Produktion" and DTU.

First and foremost I would like to thank my main supervisor Thomas Ostenfeld Larsen for giving me the opportunity to engage in the PhD study, and for his enthusiastic guidance throughout the project. Memorable journeys to New Zealand and California were great scientific experiences, and so was the recreational bungy jumping and killing old kiwi rental cars up Baldwin St.

I also owe my co-supervisors, Jens Christian Frisvad and Charlotte Held Gotfredsen, much gratitude for their ever open doors for a lost PhD student, and invaluable help during my study. A significant part of my work was based on mass spectrometry and for answers and discussions of every aspect of these instruments and data, as well as many other things; I thank Kristian Fog Nielsen wholeheartedly. A special thanks to Michael Edberg Adsetts Hansen for continuing the intricate analysis of the DIMS data, even after his departure from the University and moving to South Korea.

Many other people at the Center deserve my acknowledgement for their great work and help; particularly Hanne Jakobsen, Lisette Knoth-Nielsen and Ellen Kirstine Lyhne for their enormous efforts during the last couple of years, not at least regarding the DIMS study. Richard Kerry Phipps has been an invaluable help in the laboratory and also in various other technical and scientific aspects.
I would also like to thank Marie Louise Klejnstrup for a great collaboration at the end of my study.

My collaboration with Jakob Blæsbjerg Nielsen, Michael Lynge Nielsen and Uffe Mortensen on chemical analysis of *E. nidulans* mutant strains is also highly treasured, although not acknowledged in this thesis in form of papers.

Last, but not least, I owe tremendous thanks to my wife-to-be, Rikke, who on top of my PhD work, started on her own, gave birth to our son Sebastian and relieved me of many domestic duties in my frantic run to the finish line.

Christian Rank
Kongens Lyngby, December 2009

## Summary

The species in the genus *Aspergillus* are central organisms for many aspects of human lives: they compost organic material, infest crop fields and can even be pathogenic. The most important species have been genome sequenced during the last 5 years and thus enormous amounts of data are readily available for research.

One of the most significant features of these fungi is their chemical defense: they can produce an impressive array of different and often very complex secondary metabolites, which are used battling other microorganisms and insects in the ecological niches that the fungi inhabit. The understanding of these fungi and their mycotoxins are extremely important for humans in assessing potential contamination.

The genomes of the first, important aspergilli are accessible and the first comparative analyses have been reported. The bioinformatic predictions of the putatively annotated genes for secondary metabolites, outlines the chemical potential for these strains. However, the functional understanding of the fungal genes and their organization in clusters are still being improved rapidly and so are the predictive models.

The current knowledge on these topics is addressed in a review summarizing the overall correlation between the predicted secondary metabolite genes and the reported metabolites from the first genome sequenced aspergilli.

The most pathogenic *Aspergillus* species is *Aspergillus fumigatus*, which often proves fatal for immune compromised patients. Some of the chemical constituents of this species have been linked to the pathogenic behavior. The general knowledge of the *A. fumigatus* metabolome is reviewed. A newly described species *A. novofumigatus* was found closely related to *A. fumigatus*, yet with distinct morphological and phylogenetically characteristics. New metabolites were isolated and structure elucidated, which are potentially unique to this species: the meroterpenoid novofumigatonin and the benzodiazepines *epi*-aszonalenin A-C. Structural analogues are, however reported from other members of *Aspergillus* section *Fumigati* and are important chemotaxonomical additions to the global chemical potential of these species.

The carcinogenic aflatoxin precursor sterigmatocystin is found in surprisingly many different fungal species, of which some are common food contaminators. Several reported misidentifications and questionable chemical analyses have caused doubt about which species truly produce sterigmatocystin. The claimed producers were analyzed with HPLC-UV/Vis-DAD, LC-MS and LC-MS/MS for verification. In amongst the corrections, one new aflatoxin producer: *Aspergilllus*

*togoense* and one new sterigmatocystin producer: *Penicillium inflatum* (a probable *Aspergillus* species) are here reported for the first time.

The differentiation of the species within *Aspergillus* section *Flavi* is important in the risk assessment as *A. flavus* produces aflatoxin inconsistently in contrast to the high aflatoxin production found in related species. The metabolomic approach of direct injection mass spectrometry (DIMS) was evaluated for a fast method of separating these species. The model proved inappropriate for a clear clustering, "due to the very large amount of very similar data", but indications for unique biomarkers were found, such as the expected aflatoxins and the cyclopiazonic acid analogue speradine A, which is new to *A. flavus*. A correspondence analysis was performed of the data from only *A. flavus* and its domesticated variant *A. oryzae* used in biotech industries and for fermentation of traditional Asian foods. Unique groupings of metabolites were unraveled, but the diffuse separation of the species in the DIMS study was confirmed in terms of a significant chemical overlap between *A. flavus* and *A. oryzae.* One potential chemomarker for *A. flavus* was found, which apparently has not been seen in other species and could potentially be linked to the proposed unique NRPS genes for *A. flavus* compared to *A. oryzae.* The study of many different isolates of one species proved important in assessing the chemical diversity and potential. Even with the use of many different cultivation conditions, not all of a species chemical potential can be surely expressed from a single isolate.

New metabolites from the genome sequenced *A. oryzae* RIB40 was isolated and structure elucidated. The metabolite 13-desoxypaxilline is a precursor to the tremorgenic aflatrem and dideacetyl parasiticolide A and 14-deacetyl parasiticolide A are precursors to parasiticolide A. While the overall chemical profile of *A. oryzae* RIB40 differs greatly from *A. flavus*, many of the *A. flavus* biosynthetic pathways appear to be active in *A. oryzae*.

# Sammenfatning

Arterne i slægten *Aspergillus* er centrale organismer for mange facetter af menneskets liv: de komposterer organisk materiale, inficerer marker og kan være patogene. De vigtigste arter er blevet fuld genom sekventeret i løbet af de sidste 5 år og derfor er enorme mængder af data umiddelbart tilgængelig for forskning.

Et af de mest signifikante aspekter ved disse svampe, er deres kemiske forsvar: de kan producere et imponerende arsenal af forskellige og ofte meget komplekse sekundære metabolitter, der bliver brugt i kampen mod andre mikroorganismer og insekter i den økologiske niche, som de hører til i. Forståelsen af disse svampe og deres mykotoxiner er ekstremt vigtige for mennesket i vurderingen af potentiel kontaminering.

Genomerne af de første, vigtige aspergiller er tilgængelige og de første sammenlignende studier har været rapporteret. De bioinformatiske forudsigelser af mulige gener for sekundære metabolitter ridser det kemiske potentiale op for disse svampe. Dog bliver den funktionelle forståelse af svampenes gener og deres organisation i genklustre stadig hastigt forbedret, og det gør de prediktive modeller også.

Den nuværende viden om disse emner adresseres i et review, der summerer den overordnede korrelering mellem forudsagte sekundære metabolit gener og rapporterede metabolitter fra de første aspergiller.

Den mest patogene *Aspergillus* art er *Aspergillus fumigatus*, der ofte er fatal for immunkompromitterede patienter. Nogle af de kemiske komponenter i denne svamp er blevet koblet til den patogene opførsel. Den generelle viden om *A. fumigatus* metabolomet er her analyseret. En nyligt beskrevet art, *A. novofumigatus*, er blevet fundet til at være tæt relateret til *A. fumigatus*, dog stadig med tydelige morfologiske og fylogenetiske karakteristika. Nye metabolitter er her blevet isoleret og strukturopklaret, som er potentielt unikke for denne art: meroterpenoidet novofumigatonin og benzodiazepinerne *epi*-aszonalenin A-C. Strukturelle analoger er siden raporteret fra andre medlemmer af *Aspergillus* sektion *Fumigati* og de er vigtige kemotaksonomiske tilføjelser til det globale kemiske potententiale for disse svampe.

Den carcinogene aflatoxin-foreløber sterigmatocystin er fundet i overraskende mange svampearter, hvoraf nogle er typiske fødevarekontaminanter. Flere rapporterede fejlidentificeringer og tvivlsomme kemiske analyser har skabt tvivl om hvilke arter der virkelig kan producere sterigmatocystin. De påståede producenter blev analyseret med HPLC-UV/Vis-DAD, LC-MS og LC-MS/MS for verificering. Imellem korrektionerne blev en ny aflatoxin-producent *Aspergillus togoense*

og en ny sterigmatocystin producent *Penicillium inflatum* (en mulig *Aspergillus* art) rapporteret for første gang.

Differentieringen af arter indenfor *Aspergillus* sektion *Flavi* er vigtig i risikovurdering, da *A. flavus* producerer aflatoxin i varierende grad i modsætning til den høje aflatoxin produktion, man finder i andre arter. Metabolom tilgangen med direkte injektion masse spektrometri (DIMS) blev evalueret som en hurtig metode til at separere disse arter. Modellen viste sig at være uhensigtsmæssig for en klar adskillelse, på grund af en betydelig mængde meget ens data, men indikationer for unikke biomarkører blev fundet, såsom de forventede aflatoxiner og cyclopiazonic acid analogen speradine A, som er ny i *A. flavus*. En korrespondance analyse blev udført på data fra *A. flavus* og den domestikerede variant *A. oryzae*, der bruges i biotek industrien og i fermentering af traditionelle asiatiske fødevarer. Unikke grupperinger af metabolitter blev udledt, men den diffuse separation af arter i DIMS studiet blev konfirmeret ved et signifikant, kemisk overlap mellem *A. flavus* og *A. oryzae*. En potential kemomarkør for *A. flavus*, som tilsyneladende ikke er set i andre arter, blev fundet, og kunne teoretisk kobles til de foreslåede unikke NRPS gener for *A. flavus* sammenlignet med *A. oryzae*. Studiet af mange forskellige isolater af én art viste sig vigtigt for vurderingen af den kemiske diversitet og potentiale. Selv ved brug af mange forskellige væksbetingelser, er det ikke sikkert at hele artens kemiske potentiale kan udtrykkes af ét isolat.

Nye metabolitter fra den genom sekventerede *A. oryzae* RIB40 blev isoleret og strukturopklaret. Metabolitten 13-desoxypaxillin er en forløber for den tremorgene aflatrem og dedeacteyl parasiticolide A og 14-deacetyl parasiticolide A er forløbere for parasiticolide A. Men selvom den overordnede kemiske profil af *A. oryzae* RIB40 afviger meget fra *A. flavus*, er mange af de biosyntetiske veje i *A. flavus* tilsynelande også aktive i *A. oryzae*.

# List of Original Papers and Other Publications

Rank, C., Larsen, T.O. and Frisvad, J.C. Functional Systems Biology of *Aspergillus in* Machida, M., and Gomi, K. (Eds.). *Aspergillus*: Molecular Biology and Genomics, **2010**, Caister Academic Press, Hethersett, NR, UK. ISBN: 9781904455530.

Rank, C., Phipps, R.K., Harris, P., Frisvad, J.C., Gotfredsen, C.H. and Larsen, T.O. **2006**. *epi*-Aszonalenins A, B, and C from *Aspergillus novofumigatus*. *Tetrahedron Letters* 47:6099-6102.

Rank, C., Phipps, R.K., Harris, P., Fristrup, P., Larsen, T.O. and Gotfredsen, C.H. **2008**. Novofumigatonin, a new orthoester meroterpernoid from *Aspergillus novofumigatus*. *Organic Letters* 10(3):401-4.

Frisvad, J.C., Rank, C., Nielsen, K.F. and Larsen, T.O. **2009**. Metabolomics of *Aspergillus fumigatus*. *Medical Mycology* 47 Suppl. 1:53-71.

Rank, C., Nielsen, K. F., Larsen, T. O., Varga, J., Samson, R.A., and Frisvad, J. C. Distribution of sterigmatocystin in filamentous fungi. (draft)

Rank, C., Klejnstrup, M.L., Frisvad, J.C., Gotfredsen, C.H. and Larsen, T.O. Comparative chemistry of *Aspergillus oryzae* (RIB40) and *A. flavus*. (draft)

# Thesis Outline

This thesis entitled "Mapping of secondary metabolism in biotechnologically important *Aspergillus* species" engage different approaches to mapping secondary metabolites in full genome sequenced *Aspergillus* species. A major aim has been to study the phenotypes of these fungi, to address possible genotypic features.

The fungal genus *Aspergillus* is an important group of species capable of degrading organic tissue, hereunder living humans and other animals, thus causing aspergillosis, an often fatal invasion for immune compromised patients. In the battle for nutrition, fought in dirt, dead organic material, on plants and many other places, these fungi produce an impressive array of chemical metabolites to counter bacterial threats along with fungivorious insects, other fungal species and microorganisms, secure nutritional factors and trace metals and protect themselves from UV-radiation and other DNA-damaging factors. Some of these compounds are extremely toxic to man, e.g. aflatoxins are the most carcinogenic microbial compounds known.

The knowledge of these chemicals is thus important in many aspects of our daily life. The most important species have now been genome sequenced and new possibilities to understand the occurrence, regulation and expression of secondary metabolite synthase genes and fundamental use of theirse metabolite products emerge.

Objectives:

1) Review the metabolomics of the human pathogen *A. fumigatus* and investigate closely related species for novel biomarkers to chemically substantiate their recent differentiation into new species.

2) Sterigmatocystin is an extremely important secondary metabolite that in contrast to many other mycotoxins is shared among many different species. Surprisingly, the list of sterigmatocystin producers has been growing in recent years; therefore a part of this thesis work has been dedicated to investigate the distribution of this important mycotoxin in both postulated and new species, using state-of-the art mass spectrometric analytical techniques.

3) Study phenotypical differentiation, with focus on chemistry, within the two closely related species *A. flavus* and *A. oryzae*, using both global approaches for assessment of chemodiversity and selected local chemistry in order to elucidate chemical markers and indicate possible genetic linkage.

The thesis is divided into 8 chapters. Chapter 1 serves as an introduction to the current status on knowledge and perspectives regarding secondary metabolites from full genome sequenced species. The three main thesis objectives are dealt with in the following six chapters.

Chapters 2-4 present three manuscripts describing novel chemistry from *A. novofumigatus* and review metabolomics of *Aspergillus fumigatus*. The unsubmitted manuscript in chapter 5 reviews sterigmatocystin producing fungi, and presents new sterigmatocystin and aflatoxin producers.

Chapter 6 is a manuscript ready for submission of new chemistry from the industrially important *A. oryzae* RIB40 strain. Chapter 7 engages the intricate chemistry of *A. oryzae* and *A. flavus* with two different methods and appraises the chemodiversity.

Chapter 8 discusses the conclusions of the entire thesis.

# Nomenclature

| | |
|---|---|
| AFPA | *Aspergillus flavus A. parasiticus* agar |
| ANOVA | Analysis of variance |
| CA | Correspondance analysis |
| CD | Circular dichroism |
| CI | Consistency index |
| CPA | Cyclopiazonic acid |
| CYA | Czapek yeast autolysate |
| CYAS | CYA + salt |
| DAD | Diode Array Detection |
| DCM | Dichloromethane |
| DIMS | Direct injection mass spectrometry |
| DMAPP | Dimethylallylpyrophosphate |
| DMAT | dimethylallyl tryptophan synthase |
| DNA | Deoxyribonucleic acid |
| DRYES | Dichloran rose Bengal chloramphenicol agar |
| DTU | Technical University of Denmark |
| DUL | Dulaney's medium for Penicillin |
| ELSD | Evaporative light scattering detection |
| EtOAc | Ethylacetate |
| FLD | Fluorescence detection |
| GAK | Potato-carrot agar |
| GC | Gas chromatography |
| GMMS | Glucose minimal media + sorbitol |
| HPLC | High pressure liquid chromatography |
| ITS | Internal transcribed spacer |
| MEA | Malt extract autolysate |
| MeCN | Acetonitrile |
| MeOH | Methanol |
| NMR | Nuclear magnetic resonanse |
| MP | Malt peptone |
| MVA | Mevalonic acid |
| MS/MS | Tandem MS |
| NRPS | Non-ribosomal peptide synthase |
| OAT | Oatmeal |
| OCMAS | One Compound Many Strains |
| OSMAC | One Strain Many Compounds |
| OR | Optical rotation |
| PDA | Potato-dextrose agar |
| PKS | Polyketide synthase |
| RC | Rescaled consistency index |
| RP | Reverse phase |
| RT | Retention index |
| ST | Sterigmatocystin |
| TFA | Trifluoroacetic acid |
| TLC | Thin layer chromatography |
| UV | Ultraviolet |
| VCG | Vegetative compatibility group |
| WATM | Wickerhams antibiotic test medium |
| YE | Yeast extract |
| YES | Yeast extract succrose |
| YESBEE | YES bee pollen |

## Table of Contents

# Paper 1

## Functional Systems Biology of *Aspergillus*

Rank, C., Larsen, T.O. and Frisvad, J.C.

(Published in *Aspergillus*: Molecular Biology and Genomics. 2010. Masayuki Machida and Katsuya Gomi (Eds.), ISBN: 9781904455530)

## Introduction

*Aspergillus* species are important in biotechnology, food safety, mycopathology and indoor air mycology (Tamiya and Morita, 1929; Vanden Bossche, 1988; Bennett and Klich, 1992; Martinelli and Kinghorn, 1994; Powell et al., 1994; Smith, 1994; Samson et al., 2004; Goldman and Osmani, 2007; Samson and Varga, 2008). Many species have positive biotechnological uses (Arora, 2004a, 2004b; An, 2005), but those same species may also be pathogenic and may produce mycotoxins, such as ochratoxin A and fumonisins by *A. niger* in foods and feeds (Weidenbörner, 2001; Frisvad et al., 2007b).

The genomic data of important aspergilli emerging these years provide important information for many fields and allows for new ways to uncover the chemical potential of these fungi. The sequencing has showed that there are great differences between the species and that the diversity to a large extend is found in the secondary metabolite gene clusters (Rokas et al., 2007; Fedorova et al., 2008). While most of the structures are different from one species to another, many of the biosynthetic pathways are largely identical and a few metabolites are even found across the species and occasionally also in more genera.

Many bioinformatic predictions have yet to be verified chemically, but as the metabolites are being mapped to the genome, a greater understanding of the metabolome and interactions of the secondary metabolites will surface.

## -Omics

The different –omics have been explored in the recent decade, and include genomics, transcriptomics, proteomics, interactomics, metabolomics, and fluxomics; see Andersen and Nielsen (2009). These approaches have often been based on one organism (species) at a time. Several authors (Schreiber, 2005; Oprea et al., 2007; Wuster and Babu, 2008) have stated that secondary metabolites may prove to be the missing part of the central dogma, being the chemical communication between species and influencing the genome, transcriptome and more directly the phenotypic expression of other organisms.

The outwards directed part of the metabolome can be called the exometabolome (Thrane et al., 2007) and the field of exometabolomics has been less explored than many other -omics disciplines. Only a few secondary metabolites have been explored in the field of chemical ecology (Meinwald and Eisner, 2008), plant pathology and mycopathology to a great extent, whereas the “full” exometabolome and the full characterization of the morphological and chemical differentiation has only rarely been attempted. Alongside the exometabolome, the exoproteome can be described as all the proteins secreted or linked to the outer cell wall. Furthermore, the certain carbohydrates and lipids (the carbohydrome and lipidome, respectively, used for cell recognition and other membrane associated functions) add to the complexity of secreted metabolites. Collectively all these extrolites are termed the *secretome* (Box 1, Figure 1). This secretome is dependent both on the genome,

transcriptome, proteome, metabolome and regulome of the organism, and even more influenced by intrinsic and extrinsic abiotic and biotic environmental factors such as temperature, light, water activity, atmosphere, redox potential, pH, pressure, nutrition, and co-occurring organisms. The exointeractome consists of the metabolites being secreted and bound to the cell-wall which are produced in order to communicate, combat, collaborate etc. chemically between two or more organisms. They can act between organisms of the same species (hormones and quorum sensing metabolites) and between organisms of different species (antibiotics, attractors, toxins etc.). Secondary metabolites (= specific metabolites) including volatile metabolites, extracellular enzymes, cell wall bound enzymes, hydrophobins, and several others can from an ecological and functional point of view be regarded as signals, which is why they are referred to as extrolites (chemical compounds from a living organism that are outwards directed). The extrolites are generally considered to be limited to few taxa, as will be discussed further on. For a more in-depth definition of exometabolomics/exometabolites, extrolites and secondary metabolites to the different morphological structures, see Frisvad et al. (2007a, 2008).

The extrolites are biosynthesized via several different genes, organized in gene clusters and appears to be globally regulated to some extent (Keller et al., 2005). The different structures are often so uniquely constructed, that they are only found in one or very few isolates of a given species. The diversity can to some extent be predicted from the genes themselves, but for a detailed insight on structural motifs and stereochemistry, elaborate structure elucidation is inevitable. Differences in the stereochemistry of the end product may not be evident from the nucleotide sequence, but can have important implications for the effect and use of the metabolite. The complex structures, perfected through numerous evolutionary iterations to interact with host protein targets (receptors/enzymes), are highly specific. The ecological impact of this may not yet have been studied greatly, but in the pharmaceutical industry specificity and sensitivity is one of the most important issues in developing drugs.

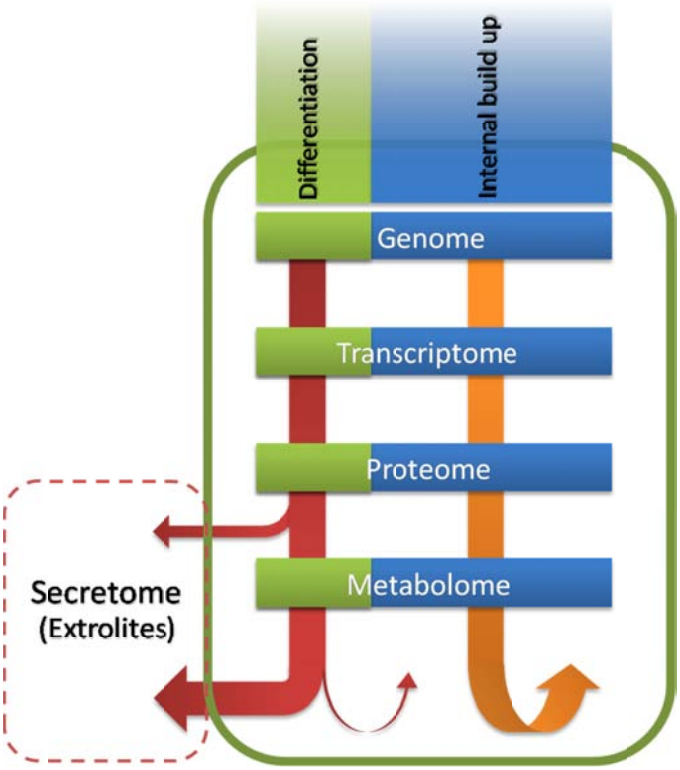


**Figure 1**: An expanded central dogma model**,** emphasizing the functional phenotype (green part) including the metabolome and the secretome/extrolites. The remaining part (blue) represents the primary metabolism and structures within the cells (for example the nucleus and membrane, the endoplasmatic reticulum, Golgi apparatus, mitochondria, actin, β-tubulin etc.).

**Box 1**. An extended central dogma model and accompanying terms.

| Differentiation | Internal cell build up | The whole organism |
|---|---|---|
| Differential genome | Endogenome | Genome |
| Differential transcriptome | Endotranscriptome | Transcriptome |
| Differential proteome | Endoproteome | Proteome |
| Differential metabolome | Endometabolome | Metabolome<br>Secretome |

| Term | | Explanation |
|---|---|---|
| **Differential genome** | | Gene clusters and regulatory genes involved in production of outwards directed chemical compounds (extrolites). |
| **Differential transcriptome** | | RNA transcripts necessary for de novo synthesis of enzymes necessary for production of extrolites, the expression of these can be silenced by histones. |
| **Differential proteome** | | The enzymes and other proteins involved in production of extrolites. |
| **Differential metabolome** | | The often unique and species specific metabolites (extrolites). |
| **Secretome** | | All outward directed chemical compounds from an organism. |
| **Extrolites** | | An ecological and chemical concept. From greek: extro- = outwards directed, ites: chemical compounds. The extrolites contain all of the concepts below and are characterized by the following functions: Hormones, quorum sensing metabolites, kairomones, allomones, toxins, attractors, adhesion molecules, and repellants. |
| | **Exoproteome** | Functional proteins secreted: extracellular enzymes, hydrophobins, bioactive proteins such as α-sarcin, mitogillin. |
| | **Exocarbohydrome** | Functional carbohydrates: difructose dianhydride, glucans, carbohydrate polymers used for biofilm production and cell recognition. |
| | **Exolipidome** | Functional lipids secreted: linoleic acid, secreted sterols. |
| | **Exometabolome** | Functional small molecules secreted: ethyl alcohol and other volatiles, citric acid, oxalic acid, gluconic acid, nucleotide (phosphate) derivatives, non-ribosomal peptides, alkaloids, terpenes, polyketides, shikimic acid derivatives, mixed origin molecules.<br>Some of these have been called secondary metabolites, specific metabolites, special metabolites, idiolites, natural products, some have formerly been called primary metabolites or general metabolites (citric acid, ethyl alcohol etc.), but when they are accumulated and secreted, they act more like secondary metabolites. |

## Aspergillus -omics

The great importance of aspergilli for industry, food, mycopathology, and toxicology has lead to the full genome sequencing of several species including *A. oryzae* (Machida et al., 2005), *A. flavus* (Payne et al., 2006), *A. fumigatus* (Nierman et al., 2005), *A. clavatus*, *N. fischeri* (Rokas et al., 2007), *Emericella nidulans* (Galagan et al., 2005; Rokas and Galagan, 2007), *A. niger* (Baker, 2006; Pel et al., 2007), and *A. terreus* (see Jones, 2007; Andersen and Nielsen, 2009). This allows for comparative genomics (Rokas et al., 2007), and recently transcriptomics (Andersen et al., 2008b; Andersen et al., 2008a; Perrin et al., 2007) has been added to the systematic examination of the genotypes of *Aspergillus* species (Andersen and Nielsen, 2009).

The latest full taxonomic monograph of the genus *Aspergillus* was written in 1965 by Raper and Fennell (1965), building on former monographs (Thom and Church, 1926; Thom and Raper, 1945), but parts of the genus have since been examined by Samson (1979), Onions et al. (1981), Samson and Pitt (1985), Kozakiewicz (1989), Tzean et al. (1990), Samson and Pitt (1990, 2000), Klich (2002), Domsch et al. (2007) and Samson and Varga (2007, 2008). Only recent taxonomic monographs have considered extrolites as part of the features that could be used in a polyphasic approach and earlier only morphology and, to a certain extent, physiology have been used in taxonomy. Later, sequencing of house-hold genes was added to the tool-box of the taxonomist.

The functional phenotype, however, has been less systematically explored. The primary metabolism is to a great extent similar in different organisms, for example similar tricarboxylic acid cycles feed primary metabolites not only to other primary metabolites, but also to the very different morphological and chemical differentiation programs of different species. Expressions of differentiation are the most important from an ecological and functional point of view, and are the base for the immense bio- and chemical diversity in *Aspergillus* species and other species of microorganisms. Differentiation in fungi can be morphological, but after all still indirectly chemical, or purely chemical. Morphological differentiation in *Aspergillus* and its teleomorphic states has two phases: an initial differentiation into those morphological elements that are in common for all *Aspergillus* species, for example the production of a “foot cell”, a stipe, a vesiculation of that stipe, the production of metulae, phialides and conidia, and in the teleopmorphic states cleistothecial structures, asci, ascospores, sclerotia, often hülle cells, followed by a supply of entirely species dependent different profiles of secondary metabolites to these morphological structures (Frisvad et al., 2007a, 2008).

### Genomic mining for functional metabolites

For studying the functional metabolome of filamentous fungi, a variety of methods can be applied. The genome sequencing has led to several new ways to discover and link new chemistry to genes.

The alleged global regulator gene *laeA* has been found to have an effect on conidial morphology for some isolates, and a profound influence on associated extrolites such as hydrophobins and secondary metabolites (Bok and Keller, 2004; Keller et al., 2005). Resently *laeA* has been shown to be involved in lightsensitive fungal development as well, emphasizing the importance of this complex part of the metabolome (Bayram et al., 2008). Control over these regulators present a novel strategy to mine the fungal genome for silent metabolites, as shown by Bok *et al.* using *laeA* control to get *E. nidulans* to produce terrequinone A (Bok et al., 2006b).
Another method for mining the genome for functional metabolites was demonstrated by Scherlach and Hertweck, when the identification of multiple anthranilic acid synthase gene copies in *E. nidulans* led them to the aspoquinolones A-D (Scherlach and Hertweck, 2006).
A third option was exemplified in our lab when we showed the expression of the fumonisins in *A. niger*:
Full genome sequencing of two different strains of *Aspergillus niger* indicated that there was a putative (and unexpected) gene cluster for fumonisins in that species (Baker, 2006; Pel et al., 2007). The reason that fumonisins have not been detected earlier in *A. niger* may have been that fumonisins were not expected to be produced by *A. niger* and furthermore their extraction and detection differ from that of most other mycotoxins. *Fusarium* species are known to produce fumonisins first of all on maize and are produced and regulated via a series of genes in addition to the actual fumonisin gene cluster (Sagaram et al., 2006; Du et al., 2008). Fumonisins are biosynthesized via a highly reduced 18-carbon polyketide coupled with two tricarboxyllic acid groups and L-alanine and methionine. An examination of three full genome sequenced *A. niger* (derived from NRRL 3, NRRL 328, and NRRL 3122) and the ex type culture of *A. niger* (NRRL 326) showed that they actually all produced fumonisins (Frisvad et al., 2007b). However, the gene clusters were not entirely similar (Baker, 2006; Pel et al., 2007) in the two groups of fungi and furthermore while *Fusarium* is a “field fungus” and *A. niger* is a “storage fungus”, it was to be expected that the regulation of fumonisin was quite different in the two types of fungi: *F. verticillioides* produced fumonisin $B_1$, $B_2$ and $B_3$ as the most dominant biosynthetic end-products, and best on agar media based on plants extracts, while *A. niger* always produce fumonisin $B_2$ as the dominant metabolite in that biosynthetic family and best on media with low water activity, such as media with high salt or carbohydrate concentration (Frisvad et al., 2007b). Thus while bioinformatics and full genome sequencing could predict potential fumonisin production in *Aspergilus niger*, phenotypic expression had to be confirmed and detected by direct exometabolome analysis.

The sequencing and subsequent annotation of the genes in aspergilli has uncovered the potential number of clusters responsible for most of the secondary metabolites. Though not yet complete and with the refinements of the prediction tools (such as SMURF developed by N. Fedorova (2008)) that will naturally follow along with the increasing understanding of the sequences, these numbers supply

us with a guide to the number and origin of the bulk set of functional molecules. The total mapping of chemistry to this genetic potential and in the future a full linkage of specific sets of metabolites to a specific genome will create the pillars on which true metabolomics may be performed. Table 1 lists the published predictions, for the full genomes of the important aspergilli, on non-ribosomal peptide synthases, responsible for extrolites incorporating more than one amino acid (NRPSs) (Mootz and Marahiel, 1997), polyketide synthases (PKSs) and dimethylallyl tryptophan synthases (DMATs). The table does not include the MVA pathway related genes; responsible for terpene biosynthesis, since only few, fully structural characterized terpenoids have been reported from aspergilli.

**Table 1**. List of predicted number of NRPSs, PKSs and DMATs genes important for the potential number of extrolites from the full sequenced aspergilli. Where a range is given, different numbers of predicted genes have been published. * refers to PKS-like genes. Empty fields mean that no total count was found in the literature. Please refer to original articles for in depth discussion of predictions.

| Species | NRPS | PKS | DMAT | References |
|---|---|---|---|---|
| *A. flavus/A. oryzae* | 24/18 | 34-35/ 30-32 | /2 | (Nierman et al., 2005; Payne et al., 2006; Rokas et al., 2007; Keller et al., 2005) |
| *A. terreus* | 22 | | | (Cramer et al., 2006) |
| *A. niger* | 17 | 34 | | (Pel et al., 2007) |
| *N. fischeri* | 19+9* | 17+1* | 10 | (Fedorova et al., 2008) |
| *A. fumigatus* | 14-13+(4;5)* | 14-13+(2;1)* | 7 | (Nierman et al., 2005; Keller et al., 2005; Cramer et al., 2006; Fedorova et al., 2008) ; indicates the two sequenced isolates Af293;A1163 |
| *A. clavatus* | 12+6* | 16+1* | 3 | (Fedorova et al., 2008) |
| *A. nidulans* | 14 | 27 | 2 | (Nierman et al., 2005; Cramer et al., 2006; Rokas and Galagan, 2007; Keller et al., 2005; Bok et al., 2006c) |

In Table 2 a list of the typical secondary metabolites from some of the recently full sequenced *Aspergillus* species divided into biosynthetic families are shown. When comparing the listed metabolites (Table 2) to

the genomic *predictions* (Table 1) it is important to remember that the sequenced genomes represent only one genotype and these data were based on many different isolates, greatly amplifying the chemical diversity, in accordance with the previously discussed functional divergence. From the comparison of the two tables it is evident that there is a lot of chemistry to be discovered especially in *Neosartorya fischeri*.

The chemistry presented in Table 2 may be classified in various ways; a more ecological approach would be to list the metabolites based on bioactivity, since this is one of their primary functions in

**Table 2**: Typical and important secondary metabolites from the full genome sequenced *Aspergillus* species. The list of metabolites is not comprehensive, but rather verified in several strains and detected in our analysis. *Since *A. flavus* is regarded as the wild type of the domesticated form *A. oryzae*, it is highly probable that extrolites from one species are also present in the other species.

| | *A. flavus* | *A. oryzae* | *A. terreus* | *A. niger* | *N. fischeri* | *A. fumigatus* | *A. clavatus* | *E. nidulans* |
|---|---|---|---|---|---|---|---|---|
| **Simple organic acids from glucose** | Kojic acid[1] | Kojic acid[1-5] | Itaconic acid[6] | Citric acid[7-9]<br>Oxalic acid[8,10] | | Epoxysuccinic acid[11,12] | | |
| **Polyketides** | Aflatoxin $B_{1+2}$[13,14]<br>(3-*O*-)-sterigmatocystin[15]<br>Versiconal hemiacetal acetate[16,17] | Asperfuran[18]<br>Maltoryzine[19] | Citreoviridins[20]<br>Geodin[21,22] Lovastatin<br>Terreic acid[23]<br>Terrein[24,25] | Aurasperone[26,27]<br>Flavones[26,27]<br>BMS-192548[28,29]<br>Fumonisins[30,31]<br>Kotanins[32-36]<br>Antafumicin[37] | Neosartorin[38] | Fumigatins[39-50]<br>Trypacidins[51-56]<br>Melanins[57-59]<br>Pseurotins[60-65]<br>Sphingofungins[66,67] | Kotanin[32-35]<br>Patulin[68-71]<br>Cytochalasin[72,73]<br>Antafumicin[37] | Asperthecin[74,75]<br>psiA[76,77]<br>Sterigmatocystin[78-80] |
| **Terpenes** | *Sporogen AO 1[81,82] | Sporogen AO 1[81,82] | Terretonins[83,84] | Asperfuranone[85,86] | | Helvolic acid[64,87-92]<br>Fumagillin[93-97] | | |
| **NRPS/Peptides** | *Aspergillomarasmine[98]<br>Aspergillic acid[131,132]<br>Aspirochlorines[99-101]<br>cyclo(D-N-Methyl-Leu-L-Trp)[99]<br>Tryptophenaline[102] | Aspergillomarasmine[98]<br>Aspirochlorines[100,101,103] | Acetylaranotin[104]<br>Asteroxepin[105]<br>Terramides[106] | Anafp[107] | | Asp F1[108,109]<br>Gliotoxin[95,110-118]<br>Mitogillin[108]<br>Restrictocins[119] | α-sarcin[120] | Penicillin[121,122] |
| **Mixed biosynthesis** | Shikimic acid +terpene:<br>CPA[123-127]<br>Aflavinines[128]<br>Aflatrem[129,130] | Shikimic acid +terpene:<br>CPA[133,134] | Meroterpenoid:<br>Terreulactone[135,136]<br><br>Shikimic acid +terpene:<br>Aspulvinones[137]<br>Asterrelenin[84]<br>Asterriquinone[138] | NRPS + PKS:<br>"Nigragillins"/<br>nigrazines[139,140] | Shikimic acid:<br>Fiscalins[141]<br><br>Shikimic acid +terpene:<br>Fumitremorgin[113,142-149] | Meroterpenoid:<br>Fumigatonin*[150]<br>Pyripyropenes[151-158]<br><br>Shikimic acid:<br>Fumiquinazolins[113,159,160]<br><br>Shikimic acid +terpene:<br>Fumitremorgin[113,142-149]<br>Fumigaclavines[56,161-164] | Shikimic acid:<br>Tryptoquivalines[53] | Meroterpenoid:<br>Shamixanthones[165-167]<br>Austins[168,169]<br><br>Shikimic acid +terpene:<br>Aspoquinolones[170]<br>Emerin[171] Terrequinone A[172] |

1 (Birkinshaw et al., 1931), 2 (Saito, 1907), 3 (Yabuta, 1912), 4 (Yabuta, 1913), 5 (Yabuta, 1916), 6 (Calam et al., 1939), 7 (Butkewitsch, 1923), 8 (Wehmer, 1924), 9 (Porges, 1932), 10 (Raistrick and Clark, 1919), 11 (Martin and Foster, 1955), 12 (Wilkoff and Martin, 1963), 13 (Sargeant et al., 1961), 14 (Asao et al., 1963), 15 (Burkhard and Forgacs, 1968), 16 (Fitzell et al., 1977), 17 (Steyn et al., 1979), 18 (Pfefferle et al., 1990), 19 (Iizuka and Iida, 1962), 20 (Sakabe et al., 1964), 21 (Raistrick and Smith, 1936), 22 (Clutterbuck et al., 1937a), 23 (Sheehan et al., 1958), 24 (Raistrick and Smith, 1935), 25 (Clutterbuck et al., 1937b), 26 (Ganguly and Sarre, 1970), 27 (Tobe et al., 1976), 28 (Kodukula et al., 1995), 29 (Shu et al., 1995), 30 (Pel et al., 2007), 31 (Frisvad et al., 2007b), 32 (Büchi et al., 1971), 33 (Büchi et al., 1977), 34 (Cutler et al., 1979), 35 (Nielsen et al., 1999), 36 (Huttel and Muller, 2007), 37 (Fujimoto et al., 1993), 38 (Proksa et al., 1998), 39 (Anslow and Raistrick, 1938a), 40 (Anslow and Raistrick, 1938b), 41 (Pettersson, 1963a), 42 (Pettersson, 1963b), 43 (Pettersson, 1964a), 44 (Pettersson, 1964c), 45 (Pettersson, 1964b), 46 (Pettersson, 1964c), 47 (Pettersson, 1965), 48 (Yamamoto et al., 1965), 49 (Yamamoto et al., 1970), 50 (Packter and Glover, 1965), 51 (Turner, 1965), 52 (Afzal et al., 1969), 53 (Balan et al., 1963), 54 (Balan et al., 1964), 55 (Balan et al., 1965), 56 (Liu et al., 2004), 57 (Watanabe et al., 2000), 58 (Tsai et al., 2001), 59 (Youngchim et al., 2004), 60 (Bloch et al., 1976), 61 (Weber et al., 1976), 62 (Bloch and Tamm, 1981), 63 (Breitenstein et al., 1981), 64 (Ando et al., 1991), 65 (Wenke et al., 1993), 66 (Vanmiddlesworth et al., 1992a), 67 (Vanmiddlesworth et al., 1992b), 68 (Wiesner, 1942), 69 (Bergel et al., 1943), 70 (Bergel et al., 1944), 71 (Varga et al., 2007), 72 (Steyn et al., 1982), 73 (Takamatsu et al., 2002), 74 (Neelakantan et al., 1957), 75 (Birkinshaw and Gourlay, 1961), 76 (Mazur et al., 1990), 77 (Mazur et al., 1991), 78 (Aucamp and Holzapfe, 1970), 79 (Pachler et al., 1976), 80 (Cox and Cole, 1977), 81 (Tanaka et al., 1984a), 82 (Tanaka et al., 1984b), 83 (Kosemura et al., 1992), 84 (Li et al., 2005), 85 (Jefferson, 1967b), 86 (Jefferson, 1967a), 87 (Chain et al., 1943), 88 (Menzel et al., 1944), 89 (Cram and Allinger, 1956), 90 (Allinger and Coke, 1961), 91 (Iwasaki et al., 1970), 92 (Okuda et al., 1964), 93 (Eble and Hanson, 1951), 94 (Tarbell et al., 1955), 95 (Tarbell et al., 1961), 96 (Schenck et al., 1955), 97 (Mccorkindale and Sime, 1961), 98 (Haenni et al., 1965), 99 (Klausmeyer et al., 2005), 100 (Sakata et al., 1982), 101 (Sakata et al., 1987), 102 (Springer et al., 1977), 103 (Sakata et al., 1983), 104 (Nagaraja et al., 1968), 105 (Kirby et al., 1986), 106 (Garson et al., 1986), 107 (Lee et al., 1999), 108 (Arruda et al., 1990), 109 (Arruda et al., 1991), 110 (Johnson et al., 1943), 111 (Beecham et al., 1966), 112 (Waring et al., 1986), 113 (Afiyatullov et al., 2005), 114 (Johnson et al., 1953), 115 (Johnson and Buchanan, 1953), 116 (Waring et al., 1987), 117 (Waring and Beaver, 1996), 118 (Kirby et al., 1988), 119 (Yang and Kenealy, 1992), 120 (Varga and Samson, 2008), 121 (Dulaney, 1947a), 122 (Dulaney, 1947b), 123 (Gqaleni et al., 1996), 124 (Horn and Dorner, 1999), 125 (Luk et al., 1977), 126 (Richard et al., 1992), 127 (Vinokurova et al., 2007), 128 (Gallagher et al., 1980b), 129 (Gallagher and Wilson, 1978), 130 (Gallagher et al., 1980a), 131 (Woodward, 1947), 132 (Dutcher, 1958), 133 (Matsudo and Sasaki, 1995), 134 (Orth, 1977), 135 (Kim et al., 2002), 136 (Yoo et al., 2005), 137 (Ojima et al., 1976), 138 (Yamamoto et al., 1976), 139 (Caesar et al., 1969), 140 (Isogai et al., 1975), 141 (Wong et al., 1993), 142 (Yamazaki et al., 1971), 143 (Dix et al., 1972), 144 (Yamazaki et al., 1974), 145 (Yamazaki et al., 1975b), 146 (Yamazaki et al., 1975a), 147 (Eickman et al., 1975), 148 (Afiyatullov et al., 2004), 149 (Abraham and Arfmann, 1990), 150 (Okuyama et al., 1984), 151 (Omura et al., 1993), 152 (Kim et al., 1994), 153 (Tomoda et al., 1994a), 154 (Tomoda et al., 1994b), 155 (Jeong et al., 1994), 156 (Tomoda et al., 1995), 157 (Tomoda et al., 1996b), 158 (Tomoda et al., 1996a), 159 (Numata et al., 1992), 160 (Takahashi et al., 1995), 161 (Spilsbury and Wilkinson, 1961), 162 (Cole et al., 1977a), 163 (Cole et al., 1977b), 164 (Flieger et al., 1997), 165 (Chexal et al., 1975), 166 (Ishida et al., 1976), 167 (Ishida et al., 1978), 168 (Scott et al., 1985), 169 (Mcintyre et al., 1986), 170 (Scherlach and Hertweck, 2006), 171 (Ishida et al., 1972), 172 (Bok et al., 2006b).

nature. The level of information on the bioactivity is, however, somewhat random and it can be difficult to estimate the *in vivo* function.
New tools to overcome the difficult manipulation of asexual aspergilli, recently developed by J.B. Nielsen *et al.* (Nielsen et al., 2008), will allow for a highly efficient gene targeting. Here silenced gene clusters are an important target for extrolites that are difficult to sample using normal growth conditions (Peric-Concha and Long, 2003; Nielsen and Oliver, 2005). This strategy has recently led to discovery of novel PKS-NRPS hybrid metabolites from *E. nidulans* (Bergmann et al., 2007).

It is well known that the *in vitro* exometabolome profile of *Aspergillus* is largely affected by the nutrients and trace metals added to the growth media, however, little work has been published on the *in vivo* growth and chemical profiles. Essential plant oils have been shown to regulate the growth of *Aspergillus* section *Flavi* on crops (Dikbas et al., 2008; Bluma et al., 2008). *Aspergillus fumigatus* produces gliotoxin, a suspected co-virulence factor in invasive aspergillosis, which has been found in both *in vitro* and *in vivo* samples (Lewis et al., 2005a; Kupfahl et al., 2008). *A. fumigatus* has only recently been shown to be differently regulated during *in vivo* growth in humans and with the special growth conditions present in the tissue, gliotoxin was again found as an important part of the invasive pathogenicity, possibly alongside other unidentified functional metabolites (Gravelat et al., 2008).

## Methods - Extrolite Analysis

In general, metabolomics refers to the sampling, analysis and identification of all low molecular mass organic compounds synthesized and modified by a living cell or organism (Fiehn, 2002). Though the secretome only represents a part of the metabolome (leaving out primary metabolites that need to be sampled using quenching approaches (Villas-Boas et al., 2005), different analytical approaches and methods are still needed for analysis targeting all the chemically diverse types of extrolites, including both volatile and non-volatile secondary metabolites. If the functional metabolites are to be coupled to the genome, great care must be taken in sampling data systematically and coherently. Bino *et al*. listed a set of criteria for compound analysis and identification for functional genomics comparisons (Bino et al., 2004).

### Sampling and instrumentation

Fungal volatile extrolites such as mono-, sesqui- and diterpenoids (Larsen and Frisvad, 1995a), that are released to the surroundings (headspace) for example during spore formation, can be sampled by either passive or active sampling using headspace techniques, such as solid phase microextraction (SPME) and then analysed by gas chromatography coupled to mass spectrometry (GC-MS) (Larsen and Frisvad, 1994; Nilsson et al., 1996). Volatiles not yet liberated from the fungal biomass can be extracted by extraction or steam distillation and extraction (Larsen and Frisvad, 1995b).

Non-volatile exometabolites are usually extracted using different solvents. The choice of solvent(s) depends on whether specific or whole profiles of metabolites are targeted. E.g. for sampling of very polar mycotoxins such as fumonisins, water needs to be used together with an organic solvent such as acetonitrile to achieve an effective extraction (Frisvad et al., 2007b), whereas mixtures of methanol/ethylacetate/dichloromethane have proven to be excellent for a more broad extraction of both polar (small polyketides such as patulin), "midpolar" (larger polyketides (such as aflatoxins) and alkaloids (such as fumiquinazolines)) and more apolar metabolites (ergosterol). Subsequently, the fungal extracts dissolved in UV transparent solvents such as methanol or acetonitrile can be analyzed by hyphenated techniques using liquid chromatography coupled to diode array detection (DAD) covering UV and VIS light absorption, fluorescence detection (FLD), nuclear magnetic resonance, mass spectrometry (MS), and/or evaporative light scattering detection (ELSD), the latter two being destructive detection methods (Larsen et al., 2005b).

## Global vs Local Chemistry

### Global chemistry

The study of the exometabolome can be dealt with in two ways: globally or locally. Our global approach is based on the metabolite fingerprinting and cluster analysis of the crude extracts of these fungi on selected media with the ability to trigger maximum chemical diversity. For fingerprinting purposes, where the aim usually is to get an overall "global chemical picture" of the sample, the signal/peaks do not necessarily need to be identified/related to specific metabolites in order to group either wild types or mutant strains into clusters.

One of the most elegant techniques for rapid fingerprinting is direct injection mass spectrometry or DIMS (Smedsgaard and Frisvad, 1996). When using mild electrospray ionization conditions numerous protonated (or sodiated), but otherwise usually non-fragmented, metabolites present in the extract can be detected in what is called a "mass profile" (Smedsgaard and Frisvad, 1996). In most cases the production of secondary metabolites is very consistent from isolate to isolate in a species as seen when comparing the mass profiles of two *A. fumigatus* extracts and two *A. lentulus* extracts (Figure 2) (Larsen et al., 2007).

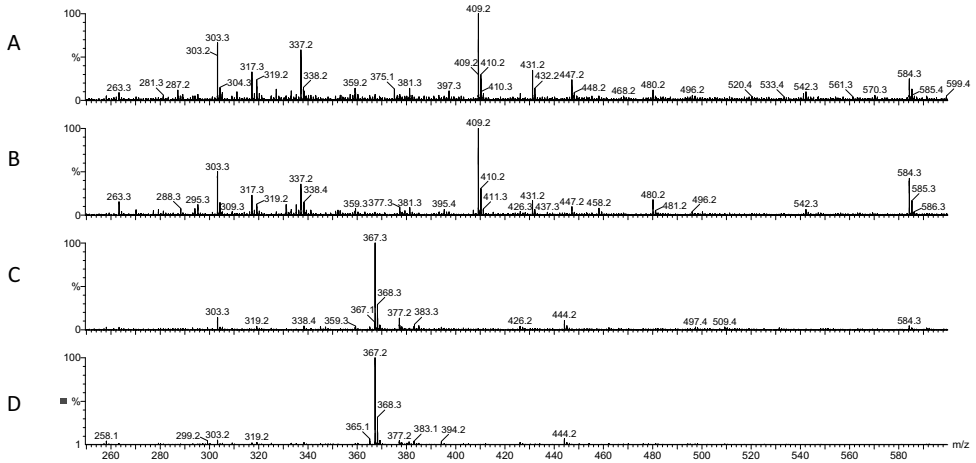


**Figure 2**. Mass profiles of *Aspergillus lentulus*, IBT 26436 (A) and IBT 23719 (B) and *A. fumigatus* IBT 24699 (C) and IBT 14904 (D). Mass profiles can be seen to be very species characteristic. The major ion peak in the mass profiles of the two *A. fumigatus* strains is likely to represent the protonated species $[M+H]^+$ of fumigaclavine C ($m_w$ = 366 Da) (Larsen et al., 2007).

This means that clustering of such mass profiles by chemometric methods usually leads to very distinct groups of extracts representing species or chemotypes of the fungi investigated as shown in Figure 9.3 (Larsen et al., 2007). The chemotaxonomical approach has been well documented as an alternative to phylogenetic methods and cluster analysis of the exo-metabolome has been shown to be a strong method for differentiating aspergilli to species level (Smedsgaard and Nielsen, 2005). The method it often more coherent than phylogenetic sequences, since it is based on patterns more than specific markers, e.g. “house-hold genes”, such as the much conserved β-tubulin, calmodulin and actin genes (Geiser et al., 1998). The chemotaxonomic approach is also used for phenotypic based drug discovery purposes (Larsen et al., 2005b).

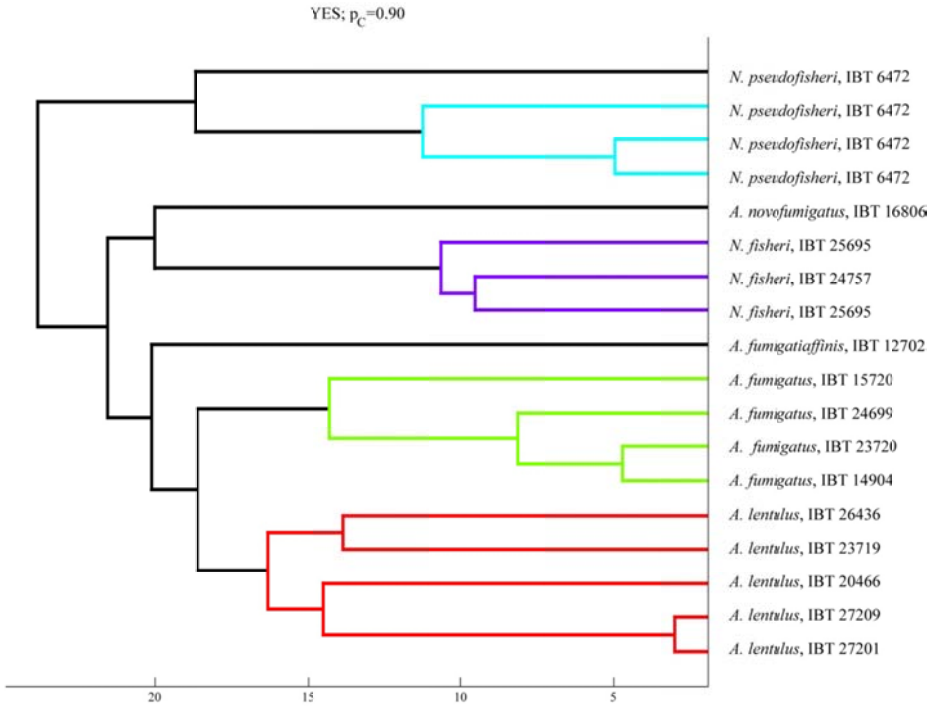


**Figure 3**. Cluster analysis based on direct injection mass spectrometric analysis of *Aspergillus fumigatus* and related species. All species are clearly separated and especially the new species *A. novofumigatus* and *A. fumigatiaffinis* are separated from the other species (Larsen et al., 2007) (reprinted with permission from *Medical Mycology*).

**Local chemistry**

Looking into the local chemistry means identification of individual metabolites seen as e.g. peaks in a metabolite profile (usually a chromatogram) where e.g. DAD and MS have been used in hyphenation. When dealing with identification of a given metabolite it is of utmost importance to identify already known compounds as quickly as possible - this process is referred to as dereplication. A thorough dereplication process will ensure that isolation and characterization of apparently unknown compounds can be focused on true unknowns. For dereplication MS and especially high resolution MS is the core technology, when used in combination with databases such as *SciFinder* (2008), *Antibase2008* (Laatsch, 2008) and *MarinLit* (Blunt and Munro, 2008) however, data generated by DAD often give important and complementary structural information since many secondary metabolites (such as polyketides and aromatic non-ribosomal peptides) contain conjugated double bonds which give rise to very characteristic UV spectra that can be searched for automatically. UV based analysis is especially well suited for relating several metabolites in a given biosynthetic

pathway, since such metabolites (e.g. pyripyropenes) share practically identical UV spectra. For more details on MS and UV-based dereplication, please consult some of the many papers and chapters that our group has published on this topic during the last five years (Nielsen and Smedsgaard, 2003; Hansen et al., 2005; Larsen et al., 2005b; Larsen et al., 2005a; Larsen and Hansen, 2007).

## General *Aspergillus* Chemistry

One of the first comparative studies of the aspergilli genomes showed that the homology between *A. fumigatus* and *A. clavatus* proteins is roughly that of humans and birds (Fedorova et al., 2008). This profound molecular divergence is especially expressed in the secondary metabolites for the various *Aspergillus* species. Table 2 lists some of the most important and representative extrolites for the genome sequenced genera. Though there are only few genuine cross-species repeats, some compound classes such as the anthraquinones (e.g. emodin and norsolorinic) and epipolythiodioxopiperazines (e.g. gliotoxins and aspirochlorines) are more broadly distributed.
Incorporation of tryptophan is another commmen motif of the extrolites known from the aspergilli. Aflavinines, aflatrem, aszonalenins, tremorgins, cyclopiazonic acid, echinulins, fumigaclavines, fumiquinazolines, paspalinines, asterriquinones and many others share the incorporation of this shikimic acid derived precursor metabolite.

**New species *A. novofumigatus* vs *A. fumigatus***

Species specific biomarkers are not to be used unambiguously for species differentiation for reasons explained previously, but rather as a final differentiation between closely related species: aflatoxin $B_1$ and aflatoxin $B_2$ are for example produced by *A. flavus* whereas aflatoxin $G_1$ and $G_2$ are not. In the case of *A. novofumigatus* it is clear that despite some resemblance to *A. lentulus*, *A. fumigatus* and especially *N. fischeri* for the calmodulin and β-tubulin genes, these isolates have very few exotrolites in common (Hong et al., 2005); *epi*-aszonalenin A-C, not found in *A. fumigatus* sensu stricto, was isolated from *A. novofumigatus* (Rank et al., 2006), along with novofumigatonin (Rank et al., 2008), neosartorin and cycloechinulin. Helvolic acid is shared between *A. fumigatus* and *A. novofumigatus*, but the latter shares neosartorin with *A. lentulus* and *N. fischeri* (based on UV-data). No fumagillin, fumitremorgins, fumiquinazolins, gliotoxins, pseurotins, trypacidin or verruculogen were found in *A. novofumigatus*.

One example of a specific marker is the fumigatonins, which are meroterpenoid orthoesters that have only been reported twice: fumigatonin from an *A. fumigatus* in 1984 by Okuyama *et al.* (Okuyama et al., 1984) and novofumigatonin (Figure 9.4) from *A. novofumigatus* as mentioned above, and although being assembled from the same farnesyl-tetraketide intermediate (Simpson, 1979; Simpson and Walkinshaw, 1981), there is still a great structural diversity in the end products. A

search for either of the fumigatonins in several *A. fumigatus* and *A. lentulus* has been unsuccessful so far.

**Cross-species metabolites: cyclopiazonic acid, kojic acid, sterigmatocystin and gliotoxin.**
It is intriguing to find some of these secondary metabolites that are distributed more widely, where entire gene clusters appear to be shared between species or even between different genera, giving rise to evolutionary implications for these highly organized gene clusters. Here, four different – but very important extrolites – are examined with respect to general effects and which species are capable of synthesizing them.

**Cyclopiazonic acid**
One compound that is shared among many species is the mycotoxin α-cyclopiazonic acid (α-CPA) which originates from a mixed biosynthetic pathway. α-CPA was originally found in *Penicillium cyclopium* (Holzapfel, 1968; Frisvad, 1989) and since in several other penicillia (Hermansen et al., 1984). Holzapfel later found β-CPA, a non-cyclizised precursor for α-CPA (Holzapfel et al., 1970), and as McGrath continued Holzapfel's biosynthetic work, he found that β-CPA comes from a DMAPP addition to *cyclo*-acetoacetyl-L-tryptophanyl, which again is a product of tryptophan and acetoacetyl-CoA (Mcgrath et al., 1976, 1977). α-CPA is a good chelator of $M^{2+}$ ions, such as $Mn^{2+}$, $Mg^{2+}$ and $Cu^{2+}$ (Mcgrath et al., 1977), hepacarcinogenic when given orally and acute toxic when administrated by intraperitoneal injection, with a $LD_{50}$ of 36-63 mg/kg for rats (Purchase, 1971). The proposed reason for the lesser toxicity in oral uptake is the lower bioavailability of the protonated α-CPA below pH 7 (Purchase, 1971). This metabolite has been found in *Aspergillus* section *Flavi*: *A. flavus* (Vinokurova et al., 2007; Horn and Dorner, 1999; Richard et al., 1992; Luk et al., 1977; Gqaleni et al., 1996), *A. oryzae* (Orth, 1977; Matsudo and Sasaki, 1995), *A. tamarii* (Vinokurova et al., 2007; Goto et al., 1996; Matsudo and Sasaki, 1995; Dorner, 1983)*, A. pseudotamari*, *A. parvisclerotigenus* (Frisvad et al., 2005) and in section *Fumigati*: *A. lentulus* (Larsen et al., 2007). The production of CPA by other species such as *A. versicolor* (Vinokurova et al., 2007; Ohmomo et al., 1973), *A. fumigatus* (Vinokurova et al., 2007), and *A. phoenicis* = *A. niger* (Vinokurova et al., 2007) could not be verified (Frisvad, 1989; Samson and Varga, 2007; Samson et al., 2007).

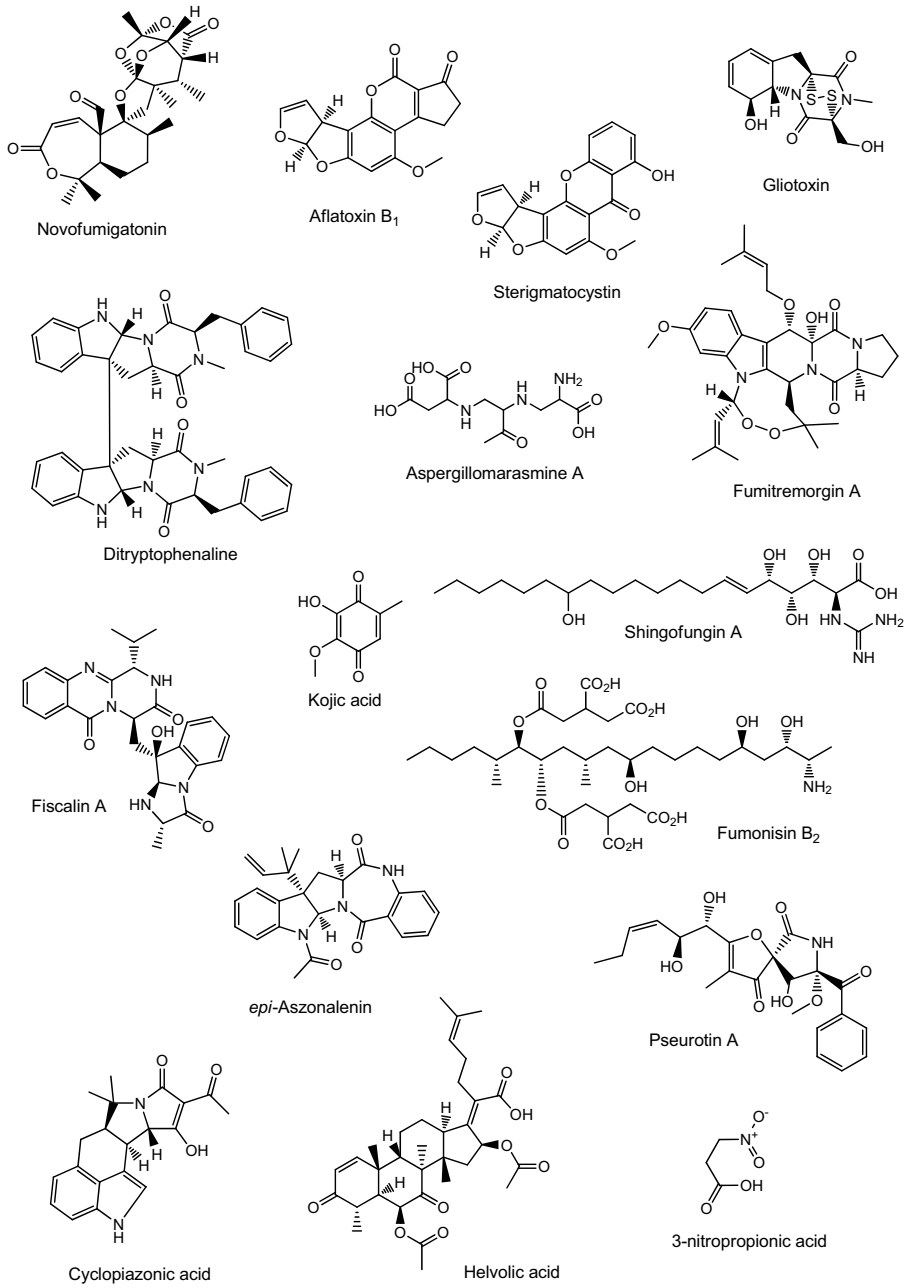


**Figure 4**. Selected extrolites illustrating the chemical diversity of *Aspergillus*.

**Kojic acid**

Kojic acid (5-hydroxy-2-hydroxymethyl-4H-pyran-4-one), a γ-pyrone derivative, is a fungal metabolite produced by many species of *Aspergillus* and *Penicillium*, as well as *Acetobacter* spp. (Terada et al., 1961). Knowledge of the compound dates back to 1907 (Saito, 1907), when Saito first began investigations. It is a good chelator of transition metal ions such as Fe (III) and Cu (II) (Beelik, 1956; Wiley *et al*. 1942). Kojic acid occurs in many fermented oriental foods (Kinosita et al., 1968), and possesses both antibacterial and antifungal activities. Knowledge about antiseptic properties dates back to studies made by Yabuta and published in 1912 (Yabuta, 1912). Kojic acid has been shown to be applicable as skin whitening agent and several patents have been granted for this purpose (Uher et al., 1993).

For more information about the kojic acid history see the review by Bentley (2006), and for a review on health aspects of kojic acid, see Burdock et al. (2001).

The long history has resulted in numerous publications of which several are erroneous. Within aspergilli, kojic acid has been reported in all species from section *Flavi*, except *A. avenaceus* (Frisvad and Samson, 2000): *A. flavus* (Parrish et al., 1966; May et al., 1931), *A. oryzae* (Beelik, 1956; Parrish et al., 1966), *A. bomycis*, *A. nomius*, *A. sojae*, *A. pseudotamarii*, *A. tamarii*, *A. caelatus* (Frisvad and Samson, 2000)*, A. parasiticus* (Parrish et al., 1966; Lin et al., 1976), *A. parvisclerotigenus*, *A. toxicarius* (Frisvad et al., 2005), *A. minisclerotigenes* and *A. arachidicola* (Pildain et al., 2008), and of course also by isolates that are synonyms of *A. oryzae* and *A. tamarii* such as *A. effusus, A. luteo-virescens, A. lutescens* and *A. gymnosardae* (Manabe et al., 1984a).

The report that *A. candidus* (= *A. albus*) can produce kojic acid (Beecham et al., 1966; Wei et al., 1991) is based on a white mutant of *Aspergillus flavus.* Species in other sections have also been reported to produce kojic acid including *A. nidulans* (Beelik, 1956; Parrish et al., 1966; Kharchenko and Yatsyshin, 1984), *A. fumigatus* (Parrish et al., 1966; Moubasher et al., 1977; Kharchenko and Yatsyshin, 1984; Kharchenko, 1993), *A. alliaceus*, *A. awamori*, *A. clavatus*, *A. giganteus*, *A. glaucus*, *A. ustus*, *A. wentii*, and *A. rubrum* by Manabe *et al.* (Manabe et al., 1984b), but these data have never been confirmed and appear to be erroneous.

**Sterigmatocystin**

Sterigmatocystin is another highly important mycotoxin found in several aspergilli, as well as in *Chaetomium* (Udagawa et al., 1979; Sekita et al., 1981; Koyama et al., 1991) and *Eurotium* species (Frisvad, 1985; Horie et al., 1989), among many other species. The metabolite is known for a close resemblance to the aflatoxins, as the biosynthetic pathway of the latter includes sterigmatocystin as a stable intermediate. Often 3-*O*-methylsterigmatocystin or 5,6-dimethylsterigmatosystin is found as the major metabolite, which is a non-aflatoxin side reaction leading to a methylated end product.

Sterigmatocystin or its methylated analogues have been reported from *A. amstelodami* (Schroeder and Kelton, 1975; Ahmed et al., 2005; Barnes et al., 1994)*, A. aurantio-brunneus* (Rabie et al., 1977),

*A. carneus* (Moubasher et al., 1977), *A. chevalieri* (Schroeder and Kelton, 1975; Ahmed et al., 2005; Moubasher et al., 1977), *A. egyptiacus* (Moubasher et al., 1977), *A. flavipes* (Moubasher et al., 1977), *A. flavus* (Schroeder and Kelton, 1975; Barnes et al., 1994; Moubasher et al., 1977), *A. fumigatus* (Hasan, 1993), *A. japonicus* (Begum and Samajpati, 2000), *A. multicolor* (Rabie et al., 1977; Hamasaki et al., 1980; Frisvad, 1985), *A. ochraceoroseus* (Klich et al., 2000; Frisvad et al., 1999), *A. parasiticus* (Schroeder and Kelton, 1975; Hsieh et al., 1973; Barnes et al., 1994; Moubasher et al., 1977; Atalla et al., 2003), *A. quadrilineatus* (Rabie et al., 1977; Barnes et al., 1994; Gbodi, 1993), *A. rambellii* (Frisvad et al., 2005), *A. ruber* (Schroeder and Kelton, 1975), *A. rugulosus* (Rabie et al., 1977; Moubasher et al., 1977; Ballantine et al., 1965), *A. stellatus* (Moubasher et al., 1977), *A. sydowii* (Wyllie and Morehouse, 1978), *A. tamarii* (Moubasher et al., 1977), *A. ustus* (Rabie et al., 1977; Moubasher et al., 1977), *A. versicolor* (Hatsuda and Kuyama, 1954; Hatsuda et al., 1954; Barnes et al., 1994; Rabie et al., 1976). However many of these reports appear to be based on misidentified isolates or to be compounds confused with sterigmatocystin. Within *Aspergillus* and teleomorphic states, the only efficient and confirmed producers of sterigmatocystin have only been found in sections *Flavi, Ochraceorosei* and *Versicolores* and *Nidulantes*. Thus confirmed producers of sterigmatocystin include *A. ochraceoroseus*, *A. rambellii*, *A. versicolor* and many *Emericella* species, while members of *Flavi* only produce sterigmatocystin transiently.

**Gliotoxin**

Gliotoxin is as mentioned earlier produced by *A. fumigatus* and the compound belongs to the class of epipolythiodioxopiperazines. Gliotoxin was first isolated from a strain of *Aspergillus fumigatus* by Johnson *et al.* (Johnson et al., 1943; Menzel et al., 1944) and later structure elucidated (Beecham et al., 1966). The less toxic bisdethiobis(methylthio)gliotoxin has also been reported from *Aspergillus fumigatus* (Waring et al., 1986; Afiyatullov et al., 2005) and so has gliotoxin G, which is a tetrasulphide analogue of gliotoxin (Waring et al., 1986). Gliotoxin has been claimed to be involved in diseases caused by *Aspergillus fumigatus* (Richard, 1997; Bok et al., 2006a; Higurashi et al., 2007; Comera et al., 2007) and contributing actively to the virulence through immunosuppresion (Kupfahl et al., 2008), likely by targeting phagocytes (Spikes et al., 2008; Comera et al., 2007).

Besides *A. fumigatus*, other medicially derived isolates of *Aspergillus* have been claimed to produce gliotoxin: *A. niger*, *A. terreus* and *A. flavus* (Lewis et al., 2005b; Kupfahl et al., 2008). However, in these cases, the closely related acetylaranotin in *Aspergillus terreus* and aspirochlorine in *A. flavus* and *A.*

*oryzae* may have been confused with gliotoxin. The production of gliotoxin by *A. terreus*, *A. flavus* and *A. niger* needs to be confirmed.

Gliotoxin is best produced on media with low amounts of carbon source. Larsen *et al.* showed that on these media all isolates of *A. fumigatus* are able to produce gliotoxin (Larsen et al., 2007).

**Functional metabolites – Sphingofungins and fumifungin**

The previous examples are some of the few extrolites found in more than one species, but the production of these functional metabolites must have an ecological origin and not a chemcial. Therefore it is interesting to compare structural resemblance rather than absolute identity. One important example of such an occurrence is the sphingofungins and fumigatonins.

The antifungal sphingofungins A-D from *A. fumigatus* ATCC 20857 (Vanmiddlesworth et al., 1992a; Vanmiddlesworth et al., 1992c) are potent and specific inhibitors of serine palmitoyl transferase, an enzyme essential in the biosynthesis of sphingolipids. *Paecilomyces variotii* produces sphingofungins E and F (Horn et al., 1992). Fumifungin (Mukhopadhyay et al., 1987) was isolated from what was probably *A. viridinutans*, as the fungus also produced viriditoxin, but sphingofungins may also be produced by *A. fumigatus* sensu stricto. These metabolites share a similar backbone to the fumonisins produced by another *Aspergillus* species, *A. niger* (Frisvad et al., 2007b) and may thus be potential inhibitors of human nerve cells. Fumonisins have been shown to cause pulmonary edema in pigs (Haschek et al., 2001) and down-regulates basal IL-8 expression in pig intestine (Bouhet et al., 2006) and therefore sphingofungins may be likely candidates to be involved in the lung infection process, also in humans.

We examined two isolates from section *Fumigati* for production of sphingofungins: the full genome sequenced *A. fumigatus* Af293 and *A. lentulus* IBT 27201. HPLC-MS data strongly indicated that both species are able to produce these compounds. A viriditoxin producer that also produced fumifungin (Mukhopadhyay et al., 1987) was probably not *A. fumigatus* or *A. lentulus*, as none of these species are able to produce viriditoxin. On the other hand, the fumifungin producing strain could have been *A. viridinutans*, *Neosartorya aurata*, or *N. denticulata* as these three species produces viriditoxin (Samson et al., 2007).

## Conclusion

The genome sequencing of important aspergilli has led to information databases containing the biological blueprints of certain isolates of important species. The information is considered absolute for these isolates, whereas the downstream information is dependent on which growth conditions were used. Before systems biology can be applied to any organism, it is essential that all possible information is mapped as the genome has been. A systematical approach to finding all metabolites and map these as potential responses from the cell, is essential for systems biology.
Mapping the secretome requires elaborate work using the many refined techniques of isolation, identification and structure elucidation. We believe the functional secretome, and the complex molecules within it, holds an untapped wealth of information that can be added to functional genomics in general and that the structure elucidation and mapping of these unique structures will aid the detailed understanding of the genomic data on the whole.

The first step towards a genuine metabolomic approach must be the full mapping of all possible metabolites; qualitative understanding as a basis for a quantitative measurement. To tap the full potential of these organisms, new approaches must be applied. Opportunities to manipulate the organisms to produce the entire scaffold of metabolites are emerging with targeting global extrolite regulators in *Aspergillus* to manipulate the production of various metabolites. Genome mining for novel chemistry is another Klondike of discoveries, waiting to be claimed.

The continued identification of novel metabolites, adding to the general knowledge on species-specific compounds is of great importance to the global metabolomic approaches. Misinterpretation of data and incorrect species identification are pitfalls for the generalization of data in metabolomic databases. Establishing bibliomes based on mining of old literature data might give unique, but erroneous, additions; literature can be an important offset for targeting expected compounds, but serious considerations about the correctness of never-reproved findings must be taken.

With the level of erroneous, uncorrected and unverified data listed in literature, it is a pitfall to build systems biology on century old information. We rather use many of these data as an inspiration than a checklist.
With dereplication, expanding structural databases, better instruments, hyphenated techniques and advanced data analysis techniques, the structural puzzle is becoming less cumbersome and the extrolites can be analyzed ever faster, leading to a full understanding of the functional systems biology of aspergilli.

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## Paper 2

### *epi*-Aszonalenins A, B, and C from *Aspergillus novofumigatus*

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# *epi*-Aszonalenins A, B, and C from *Aspergillus novofumigatus*

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**Abstract**—Three new benzodiazepines have been isolated from an unusual chemotype of *Aspergillus novofumigatus*: *epi*-aszonalenins A, B, and C. The structures were elucidated by use of one- and two-dimensional NMR spectroscopic techniques and HR ESI MS. The relative configuration was established on the basis of a single crystal X-ray diffraction study of *epi*-aszonalenin A and the absolute configuration was determined by optical rotation comparison with the literature data. The absolute configurations of *epi*-aszonalenins B and C were determined by circular dichroism comparison to *epi*-aszonalenin A.


As part of our research on industrial or otherwise important human related filamentous fungi, we have been involved in the description of the new *Aspergillus* sp. named *Aspergillus novofumigatus*.[1] Despite being closely related to *A. fumigatus*, three of the major metabolites from *A. novofumigatus* turned out to be three new variants of the known fungal metabolite aszonalenin.[2–5] The metabolites were found by the combination of the knowledge of similar compounds and the intelligent X-hitting strategy based on UV-data developed in our group.[6,7] The analysis strongly indicated that the three new compounds had a benzodiazepine-like structural motif. This was intriguing as benzodiazepines are often found to have psychoactive properties and several members of this compound class are currently in use as psychoactive drugs.[8]

*epi*-Aszonalenins A (**1**), B (**2**), and C (**3**) (Fig. 1) were isolated from an organic extract of *A. novofumigatus* grown on yeast extract sucrose (YES) agar. The extract was fractionated using C-18 reverse phase (RP) vacuum liquid chromatography and the fractions containing target molecules were selected from analytical HPLC on the basis of UV spectra, and then fractionated by preparative RP-HPLC-DAD chromatography.

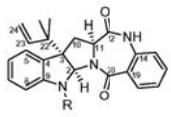


**Figure 1.** Structures of *epi*-aszonalenins A (**1**), B (**2**), and C (**3**) from *Aspergillus novofumigatus*.

*A. novofumigatus* (IBT16806) was cultured on 200 plates of YES agar at 25 °C for 14 days, and then extracted with ethyl acetate. The agar plate extract (16.2 g) was chromatographed on a Phenomenex C-18, 50 μm flash RP column using a sharp, stepped gradient from water to methanol in 10% steps. The fraction that eluted with 60% MeOH (0.66 g) was purified on a Waters HPLC column (300 × 19 mm, 15 μm, C-18), using 30 mL/min $H_2O$–$CH_3CN$ (starting at 62:38, increasing to 24:76 over 60 min) as the mobile phase to yield a mixture of **1** and **2** (180 mg combined weight).

Compounds **1** and **2** were separated on a Phenomenex Luna C-18 column (250 × 10 mm, 5 μm, C-18) using 5 mL/min $H_2O$–$CH_3CN$ (isocratic at 59:41 over 20 min) as the mobile phase to yield **1** (60 mg) and **2** (70 mg). Compound **3** was found in the fraction that

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eluted with 70% MeOH (1.38 g) and was further concentrated by fractionating through Sephadex LH-20 using MeOH as the eluent. A 50 mg portion of this extract was purified on a Phenomenex Luna C-18 column (250 × 10 mm, 5 μm, C-18) using 5 mL/min $H_2O$–$CH_3CN$ (starting at 60:40, increasing to 45:55 over 15 min) as the mobile phase to yield **3** (9.5 mg).

*epi*-Aszonalenin A (**1**) was isolated as a white solid. The structure was elucidated by a combination of NMR spectroscopy and HR ESI mass spectrometry. The HR MS[9] of **1** was consistent with a molecular formula of $C_{25}H_{25}N_3O_3$.

The NMR data used for the structure elucidation of **1** were first acquired in DMSO-$d_6$ as the solvent. The $^1$H NMR spectrum confirmed a benzodiazepine-like structure, as predicted by the X-hitting strategy. NMR-data of the compound in $CDCl_3$ were also obtained for *epi*-aszonalenin A to allow for comparison with the literature data.[3,4] The NMR-data obtained in $CDCl_3$ was less resolved than the NMR-data acquired in DMSO-$d_6$. The data for compounds **1** and **3** were almost the same as reported for acyl aszonalenin (acetyl substituent) and aszonalenin in 1982.[3] Furthermore gHMBC correlations confirmed connectivities in **1** as presented in Figure 2.

The proton shift values for H10a, H10b, and H11 in **1** however, were not the same as seen for acyl aszonalenin and more noteworthy, completely different $^3J_{HH}$ couplings were observed between the two H10's and H11 protons when compared to the literature data. Both the NMR-data from DMSO-$d_6$ and $CDCl_3$ supported these findings with almost identical values. Contrary to the two couplings of the known acyl aszonalenin, the H11 and one of the H10's of **1** show only one coupling: the doublet-H10 (designated H10a) had a coupling constant close to 13.3 Hz—a value typical of a geminal coupling. H10b is a doublet of doublets with a geminal coupling to H10a and a coupling to H11 of 9.5 Hz.

According to the Karplus equation,[10] the dihedral angle between H11 and H10a must approach ninety to minimize the *J* coupling constant, making the orbital overlap between C–H10a and C–H11 minimal. The H11 proton of aszonalenins has the opposite configuration at the bridgeheads C3 and C2 to the known aszonalenins.[2–5] Since the relative stereo geometry of the isoprene unit dictates that of C2, then H2 has to be on the same side of the two fused five-membered rings as the isoprene unit, the only variable was the configuration around C11. H11 had to point in the same direction as the isoprene unit. Figure 3 presents this difference in a Newman projection.

A series of NOE experiments were conducted in DMSO-$d_6$ to establish the stereo geometry of C11. Expected correlations between H11 and H2 were vaguely observed with mixing times above 400 ms, but this could originate from spin diffusion through H10b and the two methyl groups of the isoprene unit (C25 and C26).

X-ray crystallography supported the argument for **1** by providing a conformation for *epi*-aszonalenin A that was in agreement with the documented $^1$H NMR coupling constants and hence different from acyl aszonalenin. The X-ray structure showed disorder around the C3–C22 bond, so that two different orientations of the isoprenoid moiety were present. Figure 4 presents one of these conformations.

*epi*-Aszonalenin B (**2**) was found to be a white solid and its HR MS[12] was consistent with a molecular formula of $C_{24}H_{23}N_3O_3$. NMR data revealed that the structure only varied at the substituent on N1. It contained a

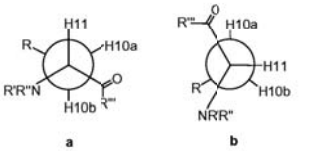


**Figure 3.** Newman projections of (a) C11–C10 of acyl aszonalenin and (b) C11–C10 of *epi*-aszonalenin A. (a) is a simplification of a MM2 model and (b) is based on the X-ray structure. In (a) the geometry of the protons on the two carbons will give rise to coupling between H11 and both of the H10's. The geometry presented in (b) will contrary to (a) give only one large coupling for H11–H10b (ideally), since in this configuration H10a is orthogonal to H11. The measured dihedral angles from X-ray analysis were: H11–H10a: 105.7° and H11–H10b: −9.9°.

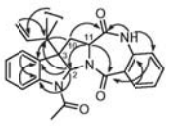

**Figure 2.** Important gHMBC correlations observed for compound **1**.

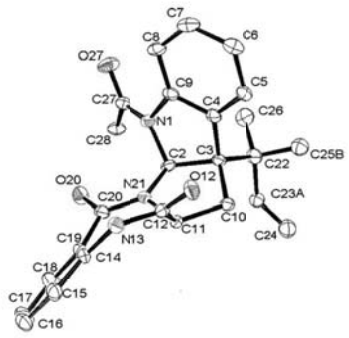


**Figure 4.** X-ray structure of *epi*-aszonalenin A[11]

**Table 1.** NMR data of *epi*-aszonalenins A (**1**),[a] B (**2**),[b] and C (**3**)[b]

| | *epi*-Aszonalenin A (**1**) | | *epi*-Aszonalenin B (**2**) | | *epi*-Aszonalenin C (**3**) | |
|---|---|---|---|---|---|---|
| | $\delta_H$ multiplicity (*J* in Hz) | $\delta_C$ | $\delta_H$ multiplicity (*J* in Hz) | $\delta_C$ | $\delta_H$ multiplicity (*J* in Hz) | $\delta_C$ |
| 1 | — | — | — | — | 6.35 br s | — |
| 2 | 6.05 s | 80.9 | 6.27 s | 78.5 | 5.55 s | 78.9 |
| 3 | — | 60.6 | — | 60.3 | — | 60.4 |
| 4 | — | 132.9 | — | 133.2 | — | 129.4 |
| 5 | 7.45 br d (7.8) | 127.3 | 7.47 br d (7.6) | 126.5 | 7.12 d (7.6) | 125.3 |
| 6 | 7.09 dt (7.8, 1.0) | 123.3 | 7.13 dt (7.6, 1.0) | 123.6 | 6.60 t (7.6) | 116.6 |
| 7 | 7.19 dt (7.8, 1.0) | 127.8 | 7.22 dt (7.6, 1.0) | 127.9 | 6.93 t (7.6) | 127.3 |
| 8 | 7.65 br d (7.8) | 117.9 | 7.69 br d (7.6) | 115.2 | 6.48 d (7.6) | 108.2 |
| 9 | — | 141.5 | — | 139.3 | — | 149.2 |
| 10a | 2.99 d (13.2) | 28.9 | 2.96 d (13.3) | 29.7 | 2.89 dd (13.5, 1.4) | 31.1 |
| 10b | 2.56 dd (13.2, 9.5) | | 2.58 dd (13.3, 9.4) | | 2.53 dd (13.5, 9.7) | |
| 11 | 4.26 d (9.5) | 56.7 | 4.36 d (9.4) | 56.5 | 4.23 d (9.7, 1.4) | 56.2 |
| 12 | — | 170.6 | — | 170.1 | — | 170.2 |
| 13 | 10.10 s | — | 10.14 s | — | 10.10 s | — |
| 14 | — | 137.0 | — | 137.0 | — | 136.6 |
| 15 | 6.97 br d (7.9) | 120.9 | 7.02 br d (7.9) | 120.9 | 7.03 br d (7.8) | 120.7 |
| 16 | 7.49 dt (7.9, 1.4) | 132.7 | 7.51 dt (7.9, 1.4) | 132.7 | 7.49 dt (7.8; 1.3) | 132.1 |
| 17 | 7.20 br t (7.9) | 123.6 | 7.23 dt (7.9, 1.0) | 123.4 | 7.21 br t (7.8) | 123.2 |
| 18 | 7.86 dd (7.9, 1.4) | 130.8 | 7.85 dd (7.9, 1.4) | 130.0 | 7.83 dd (7.8, 1.3) | 130.0 |
| 19 | — | 124.6 | — | 124.6 | — | 125.4 |
| 20 | — | 166.3 | — | 166.1 | — | 165.1 |
| 22 | — | 40.2 | — | 40.9 | — | 41.1 |
| 23 | 5.91 dd (17.4, 10.7) | 143.9 | 6.02 dd (17.3, 10.8) | 143.3 | 6.08 dd (17.4, 10.5) | 144.0 |
| 24 | 5.11 d (17.4) | 113.9 | 5.09 dd (17.3, 0.8) | 114.3 | 5.06 dd (17.4, 1.0) | 113.3 |
| | 5.08 d (10.7) | | 5.11 dd (10.8, 0.8) | | 5.10 dd (10.5, 1.0) | |
| 25 | 0.87 s | 22.8 | 0.92 s | 22.3 | 0.91 s | 22.2 |
| 26 | 1.11 s | 22.1 | 1.07 s | 21.6 | 1.06 s | 22.0 |
| C=O | — | 169.5 | 9.02 s | 161.9 | | |
| $CH_3$ | 2.61 s | 23.4 | | | | |

[a] Acquired in DMSO-$d_6$ at 499.87 MHz ($^1$H) and 125.71 MHz ($^{13}$C), respectively.
[b] Acquired in DMSO-$d_6$ at 799.63 MHz ($^1$H) and 201.01 MHz ($^{13}$C), respectively. Both spectrometers were Varan Unity Inova. Spectra were referenced according to solvent resonances at $\delta_H = 2.50$ and $\delta_C = 39.43$ ppm, respectively. A lire broadening of 0.3 Hz was applied to the $^1$H NMR spectra.

formyl-substituent that was previously unknown for aszonalenins. Besides the change in N1-substituent, **2** had similar *J* couplings, and CD spectroscopic comparison with **1** showed almost identical data.

*epi*-Aszonalenin C (**3**) was also found to be a white solid and HR MS[13] indicated a molecular formula of $C_{23}H_{23}N_3O_2$, equivalent to the loss of CO compared to **2**. This was further supported by a simplification of the fine structure in the UV-spectrum for **3** when compared to the almost identical UV-spectra of **1** and **2**. NMR data again mostly varied around the N1-position and in this case there was no substituent. H11 of compound **3** showed an additional small coupling constant of 1.4 Hz, but this does not change the key point of the argument for the dihedral angles as shown in Figure 3.

It is plausible to expect that loss of the N1-substituent would give a larger change in the overall conformation, so the 90° dihedral angle between H11 and H10a was sufficiently obscured to give a small coupling. The changes in chemical shift values for both carbon and protons are also most noticeable in **3** compared to **1** and **2**. A comparison between the data of **3** to the data obtained by Bhat and Harrison in 1986[4] on (+)-dihydroaszonalenin (which is equal to **3** except for the saturation of the terpeniod double-bond) showed excellent coherence. The (+)-dihydroaszonalenin NMR data and optical rotation were almost identical to that of **3** and definitely different from (−)-dihydroaszonalenin and aszonalenin. CD spectroscopic comparison of **1**, **2**, and **3** confirmed that the overall stereochemistry was retained between the three.

Aszonalenins have themselves been shown to be substance P inhibitors for the human neurokinin-1 receptor[14] and one may expect a similar response for the *epi*-aszonalenin variants.

Apparently the aszonalenins are not produced by *A. fumigatus* or any of the other closely related species in section Fumigati, however, this is something that we will look further into, including the fully genome sequenced strain Af293.[15]

**Acknowledgements**

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### Supplementary data

1D $^1$H NMR of **1**, **2**, and **3** has been supplied. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2006.06.086.

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9. *epi*-Aszonalenin A (**1**)—White powder; $[\alpha]_D^{20}$ +350 (*c* 0.8 mg/ml, $CHCl_3$) $\lambda_{max}$ (log ε) 220 (5.29) nm; HRESIMS: $m/z = 416.1988$ $[M+H]^+$, calcd for $[C_{25}H_{26}N_3O_3]^+$: 416.1974. Confirmed by adducts: $[M+Na]^+$ (438), $[M+MeCN+Na]^+$ (479), $[2M+Na]^+$ (853). NMR data are described in Table 1.
10. Haasnoot, C. A. G.; de Leeuw, A. M.; Allona, C. *Tetrahedron* **1980**, *36*, 2783–2792.
11. CCDC 609641 contains the Supplementary data for this letter. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.
12. *epi*-Aszonalenin B (**2**)—White powder; $[\alpha]_D^{20}$ +386 (*c* 0.5 mg/ml, $CHCl_3$) $\lambda_{max}$ (log ε) 220 (5.47) nm; HRESIMS: $m/z = 402.1800$ $[M+H]^+$, calcd for $[C_{24}H_{24}N_3O_3]^+$: 402.1817. Confirmed by adducts $[M+Na]^+$ (424), $[M+MeCN+H]^+$ (443), $[2M+Na]^+$ (825). NMR data are described in Table 1.
13. *epi*-Aszonalenin C (**3**)—White powder; $[\alpha]_D^{20}$ +670 (*c* 0.5 mg/ml, $CHCl_3$) $\lambda_{max}$ (log ε) 218 (5.46) nm; HRESIMS: $m/z = 374.1861$ $[M+H]^+$, calcd for $[C_{23}H_{24}N_3O_2]^+$: 374.1868. Confirmed by adducts $[M+Na]^+$ (396), $[M+MeCN+H]^+$ (415), $[2M+Na]^+$ (769). NMR data are described in Table 1.
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## Paper 3

### Novofumigatonin, a new orthoester meroterpenoid from *Aspergillus novofumigatus*

*Rank, C., Phipps, R.K., Harris, P., Fristrup, P., Larsen, T.O. & Gotfredsen, C.H.*

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# Novofumigatonin, a New Orthoester Meroterpenoid from *Aspergillus novofumigatus*

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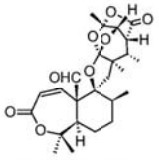
## ABSTRACT



**Novofumigatonin (1)**

**Novofumigatonin (1), a new metabolite, has been isolated from *Aspergillus novofumigatus*. The structure and relative stereochemistry were determined from HR ESI MS, one- and two-dimensional NMR, and single-crystal X-ray analysis. The absolute configuration was assigned using vibrational circular dichroism in combination with density functional calculations.**

The pathogenic fungus *Aspergillus fumigatus* is known to produce a vast array of secondary metabolites. We have previously investigated a newly characterized species of this family named *A. novofumigatus*[1] and found a series of novel benzodiazepines, the *epi*-aszonalenins.[2] Further studies on this isolate have yielded an intriguing metabolite that was isolated and purified using UV-guided fractionation.

We here report the isolation and structure elucidation of the novel orthoester novofumigatonin (**1**), related to fumigatonin[3] from *A. fumigatus* (Figure 1). The isolate of *A. novofumigatus* (deposited in the IBT Culture Collection at BioCentrum-DTU, Technical University of Denmark as IBT 16806) was cultured on 200 YES agar Petri dishes at 25 °C for 14 days and extracted with ethyl acetate containing 1% $HCO_2H$.

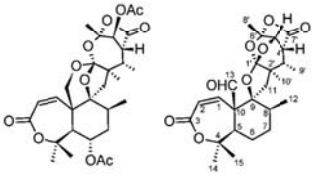


**Figure 1.** (Left) Structure of fumigatonin. (Right) Structure and numbering of novofumigatonin (1).

The agar plate extract (16.2 g) was chromatographed on flash reverse-phase columns (Phenomenex C-18, 50 $\mu$m) using a sharp, stepped gradient from water through to

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methanol. The fraction that eluted with 60% methanol (663 mg) was purified on a Waters column (300 mm × 19 mm, 15 $\mu$m, C-18), using 30 mL/min $H_2O$–$CH_3CN$ (starting at 62:38, increasing to 24:76 over 60 min) as the mobile phase to yield **1** (120 mg). Novofumigatonin **1** was obtained as a white amorphous powder. The specific rotation was measured as $[\alpha]^{20}_D$ −148° (*c* 0.04, MeOH). The UV spectrum of **1** showed a maximum end absorption at 224 nm (log $\epsilon$ 5.03). The IR spectrum confirmed this observation by revealing three distinct absorption bands in the carbonyl region at 1795, 1782, and 1703 $cm^{-1}$. The ES positive ion mass spectrum of **1** showed strong $[M + H]^+$, $[M + CH_3CN + Na]^+$, and $[2M + Na]^+$ peaks at *m/z* 461, 525, and 943, respectively. High-resolution mass measurements on the $[M + H]^+$ ion gave *m/z* 461.2203, which, in combination with $^1H$ and $^{13}C$ NMR data, suggested a molecular formula $C_{25}H_{32}O_8$ (calcd 461.2175 for $C_{25}H_{33}O_8$).

A series of DQF-COSY, TOCSY, and gHSQC experiments established four spin-systems: **a**–**d** (Figure 2). The

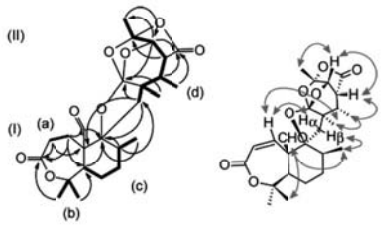


**Figure 2.** (Left) Important gHMBC correlations for fragments **I** (**a**, **b**, and **c**) and **II** (**d**) of novofumigatonin (**1**) in DMSO-$d_6$. The four spin-systems (**a**–**d**) are shown in bold. (Right) Important NOE correlations used to establish relative stereochemistry.

establishment of substructure **I**, comprised of spin-system **a**–**c**, was accomplished mainly from gHMBC correlations. Protons H1 and H2 both correlated to C3, as did methyl group $H_3$-14. The chemical shift values of C1, C2, and C4 clearly indicated that C3 is an ester carbonyl and that it was oriented with the oxygen attached to C4. H1 also showed a weak correlation to methine C5 as did $H_3$-14 and $H_3$-15. Finally, H2 correlated to the quaternary C10 and the aldehyde C13. H5 confirmed this with correlations to C4, C10, C13, and C15. H5 also correlated with the oxygen-substituted carbon C9, as did H13. H13 also correlated with C1 and C10. Spin-system **c** was elucidated as mentioned previously, and the gHMBC analysis confirmed this (see Table 1).

The rest of spin-system **c** was linked to the combined spin-systems **a** and **b** by the correlations from H8 and $H_3$-12 to C9 and H8 to C10, which confirmed the placement of the quaternary C9 and C10. A weak correlation was observed from H8 to the methylene C11 of spin-system **d**. Correlations were observed from H11 to C8, C9, and C10, which indicated placement of C11 as the linkage from fragment **I** to fragment **II**. The number of quarternary carbons in the area of this linkage, however, limited the number of gHMBC

**Table 1.** $^1H$ and $^{13}C$ NMR and HMBC Data for **1**[a]

| no. | $\delta_C$ | $\delta_H$ (mult., *J*, Hz) | HMBC correlations |
|---|---|---|---|
| 1 | 144.4 | 6.83, d, 13.1 | 2, 3, 5, 6, 9, 13 |
| 2 | 123.4 | 6.21, d, 13.1 | 1, 3, 10, 13 |
| 3 | 164.6 | – | – |
| 4 | 82.8 | – | – |
| 5 | 49.6 | 2.19, m | 4, 6, 7, 9, 10, 13, 15 |
| 6α | 18.7 | 2.14, qd, 13.2, 3.5 | 7, 10 |
| 6β | | 1.73, m | |
| 7α | 28.0 | 1.64, m | 5, 6, 8, 9, 12 |
| 7β | | 1.86, m | |
| 8 | 39.5 | 2.21, m | 6, 7, 9, 10, 11, 12 |
| 9 | 90.0 | – | – |
| 10 | 62.2 | – | – |
| 11α | 49.2 | 1.72, d, 13.8 | 8, 9, 10, 1′, 2′, 3′, 10′ |
| 11β | | 2.02, d, 13.8 | |
| 12 | 16.4 | 0.95, d, 7.2 | 7, 8, 9 |
| 13 | 201.4 | 10.26, s | 1, 9, 10 |
| 14 | 30.2 | 1.35, s | 3, 4, 5, 15 |
| 15 | 23.6 | 1.24, s | 4, 5, 14 |
| 1′ | 128.1 | – | – |
| 2′ | 46.7 | – | – |
| 3′ | 38.0 | 2.04, m | 11, 2′, 4′, 7′, 9′ |
| 4′ | 41.0 | 2.78, dd, 8.6, 6.2 | 2′, 3′, 5′, 6′, 7′ |
| 5′ | 77.6 | 5.01, d, 8.6 | 1′, 3′, 6′, 7′, 8′ |
| 6′ | 107.1 | – | – |
| 7′ | 175.1 | – | – |
| 8′ | 21.8 | 1.62, s | 5′, 6′ |
| 9′ | 11.7 | 0.99, d, 7.1 | 2′, 3′, 4′ |
| 10′ | 18.5 | 0.75, s | 11, 1′, 2′, 3′ |

[a] Data were recorded in DMSO-$d_6$ at 799.58 MHz for $^1H$ and 201.10 MHz for $^{13}C$ on a Varian Unity Inova spectrometer.

correlations. Spin-system **d** was elucidated with NMR, but the correct placement of the many oxygen bonds proved to be impossible to ascertain with NMR. Carbon C1′ was especially difficult to map.

A crystal was therefore grown in a crystallization tray from a saturated solution of 9:1 EtOH–$H_2O$. X-ray crystallographic analysis[4] revealed the remaining connectivities of **1** and also provided the relative stereogeometry as shown in Figure 3.

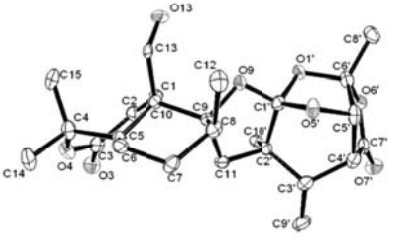


**Figure 3.** Three-dimensional structure of **1** acquired by X-ray crystallography with view point from C8 to C9. Carbon atoms in black and oxygens in red.

NOESY data for **1** (Figure 2) were consistent with the X-ray structure. The H5 proton correlated with both $H_3$-14

and $H_3$-15, but only $H_3$-15 correlated with H13. $H_3$-12 methyl group also correlated with H13. In combination with the X-ray structure, it was clear that C12, C13, and C15 were all positioned on the same side of the plane of the two rings in fragment **I**. H1 also correlated to one of the H11 protons (H11$\alpha$) and H10′. H8 and $H_3$-12 correlated to the other (H11$\beta$), and a correlation between $H_3$-12 and H3′ was also observed. $H_3$-9′ and $H_3$-10′ correlated to each other, as did H3′ with H4′. Unfortunately, all attempts to cocrystallize with heavy atom compounds were unsuccessful; thus, a vibrational circular dichroism (VCD) study was undertaken to establish the absolute configuration of **1**. VCD has recently gained importance for the determination of absolute configuration of small organic molecules,[5] especially in those cases where X-ray crystallography is impossible (e.g., liquids[6]).

A VCD spectrum is obtained as the difference in absorbance when using left- and right circularly polarized light (in the infrared region). As a direct consequence hereof, enantiomers will have spectra which are mirror images of each other. For a typical small- to medium-sized molecule there will be numerous IR absorption modes (theoretically $3N - 6$, where $N$ is the number of atoms), and the resulting VCD spectrum thus contains many absorptions which can be used to identify a particular compound. More importantly, however, the calculation of VCD spectra from first principles has matured to become a robust theoretical method which can be performed almost routinely, usually relying on density functional calculations (DFT) (B3LYP) and a relatively large basis set. This allows the determination of absolute configuration by simple comparison between experimental and theoretical spectra.

The computational investigation of **1** used the X-ray structure as the starting point, and after addition of hydrogens, the resulting structure was optimized using the OPLS-2005 force-field. This did not result in any significant changes of the structure (rms difference between the X-ray and the OPLS structure was 0.1758 Å, excluding hydrogens). Further structural optimization and calculation of optical properties (IR, VCD) was performed using density functional theory (B3LYP/6-31G**)[7,8] in Gaussian03.[9] Lorentzian lineshapes were assumed with a half-width of 4 $cm^{-1}$ in the theoretical IR and VCD spectra, and a frequency scaling factor of 0.98 was used throughout. Due to the size and complexity of **1** a perfect peak-to-peak match between theoretical and experimental spectra cannot be expected.

Accordingly, we only attempted to compare the major features in IR. Given an adequate match, VCD could be used to assign the absolute configuration of **1**. The experimental measurements were carried out by Biotools Inc. The IR and VCD spectra were recorded at a resolution of 8 $cm^{-1}$ over a period of 8 h, using a 100 $\mu$L solution of **1** (4.2 mg in $CDCl_3$).

Figure 4 shows the calculated and measured IR absorption spectra. The selected wavenumber range corresponds to

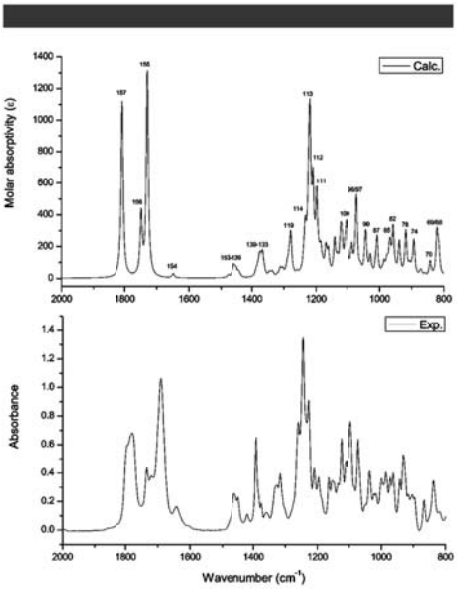


**Figure 4.** (Top) Calculated IR spectrum of novofumigatonin. (Bottom:) Experimental IR spectrum.

fundamentals 68−157 of the possible 189 for **1**. The carbonyl stretching region is significantly broader in the experimental spectrum, which may be related to coordinating solvent molecules. The inclusion of an implicit solvation model did not lead to improved results, and we deemed inclusion of explicit solvent molecules to be too computationally demanding. In the "fingerprint" region (1400−800 $cm^{-1}$) there are a multitude of vibrations associated with the skeleton of **1**.

The carbonyl stretching region did not produce useful VCD signals; thus, we have limited ourselves to the fingerprint region (1500−800 $cm^{-1}$ see Figure 5). The two most prominent peaks in the calculated spectrum (Figure 5,

(4) CCDC 670389 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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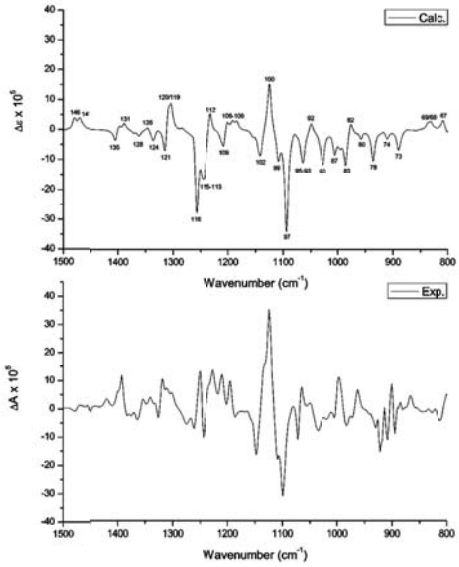


**Figure 5.** (Top) Calculated VCD spectrum of novofumigatonin. (Bottom) Experimental VCD spectrum.

top) are the positive peak resulting from fundamental no. 100 and the negative peak from fundamental no. 97.

These peaks can also be clearly identified in the experimental spectrum (Figure 5, bottom), and the good agreement allows assignment of the absolute configuration of **1** as shown in Figure 1. To assist further in the corroboration of this result we also note good agreement for peaks 102, 95–93, 92, and 90. The experimental spectrum is relatively poor in the region close to 900 $cm^{-1}$, which can be attributed to the strong absorption of the solvent ($CDCl_3$). Another discrepancy exists in the region close to 1250 $cm^{-1}$, where the calculation predicts large negative VCD absorptions (fundamentals 113–116), which cannot be found in the experimental spectrum.

A comparison of **1** to known compounds shows a high similarity to fumigatonin.[3] The major difference is the presence of an aldehyde substituent on C10 where fumigatonin has an oxygen-bound methine group and the absence of an acetate group at C6 on **1**. A closer comparison of spin-system **d** to the corresponding unit of fumigatonin also reveals that this part has the same relative stereochemistry, except for the acetate-bearing C5′ in fumigatonin. The major difference in the backbone between **1** and fumigatonin is in the linkage between fragments **I** and **II**.

The structure of fragment **I** also has some resemblance to the andilesins, fungal metabolites previously found in *Aspergillus variecolor*.[10,11] A closer comparison between fumigatonin and **1** reveals that all stereocenters have the same configuration, which strongly indicates a common biosynthetic route.

Simpson *et al.*[12] proposed that fumigatonin arises from a mixed polyketide-terpenoid pathway, similar to that of the andilesins and andiberins, but no work has been presented to date on this subject.

**Acknowledgment.** The 800 MHz spectra were obtained using the Varian Unity Inova spectrometer of the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules. The Center for Sustainable and Green Chemistry is sponsored by the Danish National Research Foundation for the period 2005–2010. The Danish Center for Scientific Computing provided computational resources.

**Supporting Information Available:** One- and two-dimensional NMR spectra of **1**, along with a full Gaussian03 reference (for ref 9), *XYZ* coordinates of the optimized structure, and enlarged IR and VCD spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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## Paper 4

### Metabolomics of *Aspergillus fumigatus*

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# Metabolomics of *Aspergillus fumigatus*

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*Aspergillus fumigatus* is the most important species in *Aspergillus* causing infective lung diseases. This species has been reported to produce a large number of extrolites, including secondary metabolites, acids, and proteins such as hydrophobins and extracellular enzymes. At least 226 potentially bioactive secondary metabolites have been reported from *A. fumigatus* that can be ordered into 24 biosynthetic families. Of these families we have detected representatives from the following families of secondary metabolites: fumigatins, fumigaclavines, fumiquinazolines, trypacidin and monomethylsulochrin, fumagillins, gliotoxins, pseurotins, chloroanthraquinones, fumitremorgins, verruculogen, helvolic acids, and pyripyropenes by HPLC with diode array detection and mass spectrometric detection. There is still doubt whether *A. fumigatus* can produce tryptoquivalins, but all isolates produce the related fumiquinazolines. We also tentatively detected sphingofungins in *A. fumigatus* Af293 and in an isolate of *A. lentulus*. The sphingofungins may have a similar role as the toxic fumonisins, found in *A. niger*. A further number of mycotoxins, including ochratoxin A, and other secondary metabolites have been reported from *A. fumigatus*, but in those cases either the fungus or its metabolite appear to be misidentified.

**Keywords** Metabolomics, *Aspergillus* section *Fumigati*, extrolites, sphingofungin

## Introduction

*Aspergillus fumigatus* is the dominating species causing fungal lung diseases in humans and animals [1–6]. Other species in *Aspergillus* section *Fumigati* and its teleomorphic (sexual) state *Neosartorya* are also able to cause aspergillosis, however. These species include *A. lentulus* [7], *N. pseudofischeri* [8,9] and *N. udagawae* [10]. While *Neosartorya* species produce both a sexual state with ascospores and an asexual state with conidiospores, the *Aspergillus* species only produce conidiospores. Several new species have recently been described in section *Fumigati* and *Neosartorya* [11–14], and an overview of the 23 species of *Neosartorya* and 10 species in *Aspergillus* section *Fumigati* is provided by Samson *et al*. [14]. It is well known that isolates of *A. fumigatus* are able to produce many extrolites [14–17], but of high importance is gliotoxin, that has been found in lungs or other infected tissues. Gliotoxin was found after experimental aspergillosis [18], and has also been found naturally occurring in turkey lungs infected with *A. fumigatus* [19]. Gliotoxin has also been found in a bovine udder infected with *A. fumigatus* [20] and in human tissues [21]. Gliotoxin may not be the only mycotoxin involved in mycosis [22] as several other extrolites have been reported from *A. fumigatus* [1,15,16,23–26]. The antibiotic fumigacin (which was later shown to be a mixture of helvolic acid and gliotoxin) has also been found in human and animal pulmonary tissues [27–30].

Two isolates of *A. fumigatus* have been full-genome sequenced and arrays are being developed for this species [31–33]. The closely related species *Neosartorya fischeri* is also being full-genome sequenced and this species and another closely related species *A. lentulus* and several new species we have described, can be compared with, and used as controls, for *A. fumigatus*, as their extrolite profiles are also known [11]. There are still extrolites that have not been identified in *A. fumigatus* and allied species [34–36], but a large

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number of extrolites are well characterized. Many extrolites from *A. fumigatus* are associated with the conidiospores, including gliotoxin, trypacidin, verruculogen and fumigaclavine A [37 41] and are thus likely to have effects in the initial lung infection process. The global regulator gene *laeA* appear to have an effect on conidial morphology, including associated extrolites such as hydrophobins and other extrolites [42–44].

It is the purpose of this paper to list, update and revise the profile of extrolites associated with *A. fumigatus* and to analyse 40 strains of *A. fumigatus* to examine the chemo-consistency in this species.

## Materials and methods

Many isolates of *Aspergillus* section *Fumigati* were examined for extrolite profiles using HPLC and MS methods, and few isolates of *A. fumigatus* and *A. lentulus* were specifically screened for sphingofungins. *A. fumigatus* isolates from different sources were emphasized (Table 1). All isolates were inoculated on Czapek yeast autolysate (CYA) agar, yeast extract sucrose (YES) agar, malt extract autolysate (MEA) agar, Oat meal (OAT) agar at 25°C and on CYA at 37°C (see Samson *et al.* [45] for formulae). Secondary metabolites were extracted from CYA and YES agar after 7 days of growth in darkness, using the extraction solvent ethyl acetate/dichloromethane/methanol (3:2:1, v/v/v) with 1% (v/v) formic acid.

HPLC with diode array detection and high resolution mass spectrometric detection (HPLC-DAD-HRMS), was performed on an Agilent 1100 system with a Luna C18 II column (Phenomenex, Torrance, CA) and equipped with a photo diode array detector (DAD), and coupled to a LCT orthogonal time-of-flight MS (Waters-Micromass, Manchester, UK), with a Z-spray ESI source and a LockSpray probe [46]. Furthermore all isolates were analyzed by HPLC-DAD using the method of Frisvad and Thrane [47,48] as modified by Smedsgaard [49].

Samples were analyzed in positive and negative electrospray interphase ($ESI^+$ and $ESI^-$) using a water-acetonitrile linear gradient system starting from 15% acetonitrile which was increased to 100% in 20 min and holding 100% for 5 min [50]. In both $ESI^+$ and $ESI^-$ two scan functions (1 s each) were used: the first with a potential difference of 14 V between the skimmers scanning m/z 100 to 900; the second with 40 V between the skimmers scanning m/z 100–2000.

Data analysis was performed as described previously [50], peaks were matched against an internal reference standard database (~730 compounds), the 33557 compounds in Antibase 2007 [51], previous data from our group [17], and the review data in this paper.

**Table 1** Isolates of *Aspergillus fumigatus* examined

| Isolate | Source |
|---|---|
| CBS 542.75 = ATCC 26606 | Man, USA |
| CBS 143.89 | Man, France |
| CBS 144.89 = CEA10* | Man, France |
| CBS 145.89 | Man, France |
| CBS 146.89 | Man, France |
| CBS 147.89 | Man, France |
| IBT Stend1 | Man, hospital Sundby, Denmark |
| IBT Stend2 | Man, hospital Hvidovre, Denmark |
| Af293* | Man, United Kingdom |
| CBS 133.61$^T$ = ATCC 1022 | Chicken lung, Connecticut, USA |
| IBT 16904 | Saltern,Slovenia |
| IBT 16902 | Saltern, Slovenia |
| IBT 16903 | Saltern, Slovenia |
| IBT 16901 | Saltern, Slovenia |
| IBT 24004 | Saltern, Slovenia |
| ATCC 32722 | Soil, Canada |
| NRRL 1979 − IBT 15720 | Soil, USA |
| CBS 113.26 = ATCC 1028 | Soil, Germany |
| CBS 132.54 − QM 6b | Soil |
| CBS 457.75 = WB 5452 | Soil, India |
| CBS 151.89 | Stone, Germany |
| CBS 152.89 | Stone, Germany |
| NRRL 5587 | ? |
| IBT D47i | Soil, Indonsia |
| CCRC 32120 | Soil, Taiwan |
| IBT 25732 | Soil under banana tree, Kenya |
| IMI 376380 = IBT 23720 | Unknown (reported ochratoxin A producer) |
| CBS 545.65 = ATCC 16913 | Unknown |
| WB 5033 − IBT 22612 | Unknown (white conidia) |
| IBT 23737 | Unknown, Denmark |
| IBT 14904 − ATCC 32722 | Unknown, USA |
| CBS 192.65 = IHEM 4392 | Feed, Netherlands |
| CBS 148.89 | Maize, France |
| CBS 149.89 | Maize, France |
| CBS 150.89 | Beetroot, France |
| IBT 21997 = A195 | Feed, Spain (reported ochratoxin A producer) |
| IBT 22023 | Silage, Germany |
| IBT PerHag | Silage, Sweden |
| IBT 22234 | Ex tea factory, Uganda |
| IBT 21711 | Food, Italy |

*Full genome sequenced strains.

## Results

The profile of extrolites produced by *A. fumigatus* has to be collected from several literature sources in

conjunction with actual metabolic profiling of a series of isolates of *A. fumigatus*. The data obtained here were verified by comparison with authentic standards, similar UV and high resolution mass spectra and literature data. We examined the isolates of *A. fumigatus* for all main secondary metabolites that have been reported in the literature, often reported from one isolate only. In this way we confirmed that isolates of *A. fumigatus* produced some secondary metabolites consistently, others by approximately half of the isolates, and some reported secondary metabolites were apparently not produced by *A. fumigatus* (Table 3). It is well known that the production of extrolites is depending on the growth medium and environmental factors [52–54], but on the media Czapek yeast autolysate (CYA) agar and yeast extract sucrose (YES) agar a large number of these representatives of the 24 families of extrolites are detectable using HPLC with diode array detection [24]. The extrolites most consistently produced were fumiquinazolines A/B, C/D and F/G (100%), gliotoxin (38%), fumigaclavine C (100%), fumitremorgins (100%), fumagillin (100%), helvolic acid (98%), pseurotin A (100%), fumigatins (35%), chloroanthraquinones (70%), melanins (100%, only verified, however, by observing that the bluegreen pigment is produced by all the isolates), and pyripyropenes (48%) (Table 3). The growth conditions and the incubation time chosen may not have been optimal for production of all extrolites by *A. fumigatus*.

*(1) Epoxysuccinic acid and difructosedianhydride (tricarboxylic acid cycle)*

Epoxysuccinic acid seems to be the major organic acid produced by *A. fumigatus* [55,56], whereas production of citric acid appears to be weak [57]. The role of epoxysuccinic acid in the life cycle of *A. fumigatus* is unknown. Atypical carbohydrates, such as difructose dianhydride may also be produced by *A. fumigatus* [58].

We did not examine any of the cultures for these two metabolites, as the detection method was not adequate for these particular metabolites.

*(2) Fumigatins (polyketides)*

The fumigatins and spinulosins consist of at least 21 polyketide extrolites (Table 1) and have been thoroughly studied concerning their biosynthesis [59–69]. Fumigatin and spinulosin are reported to be antibiotically active against several gram-negative and gram-positive bacteria [23,28] and fumigatin was cited to be toxic against experimental animals by Austwick [1], while Cole and Cox [23] claimed that vertebrate toxicity was unknown. However, later it was shown that fumiquinones A and B, in the same biosynthetic family, are toxic to other kinds of animals (nematodes) [70]. Antibacterial and antinematodal activity of the fumigatins and spinulosins can maybe explain their prevalence in soil isolates of *A. fumigatus*.

We found fumigatin in several isolates (35%), but it was most common in soil-borne *A. fumigatus* (Table 3). However, one isolate from a patient produced fumigatin.

*(3) Trypacidins (polyketides, nitrogen in one extrolite)*

Trypacidin [71–73] and monomethylsulochrin [15,71,73] were isolated and their structure elucidated by Turner [71] and Balan *et al.* [73]. Trypacidin is antiprotozoal and also an antimicrobial antibiotic [37,74]. Two other related extrolites, asperfumin, and the nitrogen containing asperfumoid has also been detected in *A. fumigatus* [15]. Trypacidin and monomethylsulochrin has been found in all isolates examined of *A. fumigatus* [11].

Most isolates (75%) examined by us produced trypacidin and monomethylsulochrin (Table 3). Isolates producing these metabolites also produced the chloroanthraquinones (Table 3).

*(4) Chloro-anthraquinones or –anthrones (polyketides)*

Emodin, physcion [15], 2-chloro-emodin, 2-chloro-citreorosein, 2-chloro-1,3,8-trihydroxy-6-methyl-9-anthrone, and 2-chloro-1,3,8-trihydroxy-6-hydroxymethyl-9-anthrone have been reported from *A. fumigatus* [75]. These polyketides have not been reported to have a role in the infection process.

We found UV spectrum evidence for production of several of the anthraquinone metabolites in many strains of *A. fumigatus* (Table 3).

*(5) Melanins (polyketides)*

*Aspergillus fumigatus* is able to produce polyketide derived melanins via a heptaketide shortening from YWA1 to 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN), which is the basis for production of 1,8-dihydroxynaphthalene, the pentaketide compound that will polymerize to melanin [76–78], giving *A. fumigatus* the green conidium colour. Melanin has been mentioned as one of several potential virulence factors in *A. fumigatus* [79,80].

As all isolated had blue-green conidia, they probably have the ability to produce this 1,8-dihydroxynaphthalene derived polymer.

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*(6) Sphingofungins and fumifungin (polyketides + alanine)*

The sphingofungins A-D from *A. fumigatus* ATCC 20857 (from soil in Uruguay) are antifungal agents [81,82] and are potent and specific inhibitors of serine palmitoyl transferase, an enzyme essential in the the biosynthesis of sphingolipids. *Paecilomyces variotii* produces sphingofungins E and F [83]. Fumifungin [84] was isolated from what was probably *A. viridinutans*, as the fungus also produced viriditoxin, but sphingofungins may also be produced by *A. fumigatus sensu stricto*. These metabolites share a similar backbone to the carcinogenic mycotoxins the fumonisins produced by *Fusarium* species and an *Aspergillus* species, *A. niger* [85] and may thus be potential inhibitors of human nerve cells. Fumonisins have been shown to cause pulmonary edema in pigs [86] and down-regulates basal IL-8 expression in pig intestine [87] and therefore sphingofungins may be likely candidates to be involved in the lung infection process, also in humans.

We examined two isolates from section *Fumigati* for production of sphingofungins: the full genome sequenced *A. fumigatus* Af293 and *A. lentulus* IBT 27201. HPLC-MS data strongly indicated that both species are able to produce these compounds (Fig. 2). A viriditoxin producer that also produced fumifungin [84] was probably not *A. fumigatus* or *A. lentulus*, as none of these species are able to produce viriditoxin. The fumifungin producing strain could have been *A. viridinutans*, *Neosartorya aurata*, or *N. denticulata* as these three species produces viriditoxin [14].

*(7) Pseurotins (mixed biosynthetic origin: polyketide + phenylalanine)*

The pseurotins were first isolated from *Pseudeurotium ovalis* (pseurotins A, B, C, D and E) [88–91], but were later isolated from *A. fumigatus* (pseurotin A, 8-O-demethylpseurotin, pseurotin F1 and F2 and synerazol) [92,93]. These compounds are chitin synthase inhibitors, but only the epoxy-pseurotin, synerazol, has antifungal activity. It is not known whether the pseurotins have biological activities of relevance for the lung infection process. The closely related compound azaspirene has been isolated from a *Neosartorya* species [94].

We found that pseurotin A was produced by all 40 strains examined of *A. fumigatus*, but some additional pseurotins, as identified based on UV-VIS spectra, were often produced at the same time.

*(8) Ergosterols (triterpenes)*

Apart from ergosterol, produced by all fungi, *A. fumigatus* has been reported to produce ergosterolpalmitat, ergosterolperoxide [95], ergosta-4,6,8(14),22. -tetraen-3-one, ergosta-4,22-diene-3β-ol, 5α,8α-epidioxy-ergosta-6,22-diene-3β-ol [15]. Ergosterolperoxide has some antiviral properties [96].

We found ergosterol in all 40 isolates of *A. fumigatus* examined, but did not screen for the other ergosterol derived compounds.

*(9) Helvolic acids (triterpenes)*

Helvolic acid [15,97–101] is an antibiotic that is active against both gram-positive and gram-negative bacteria. Other products in the biosynthetic family, such as helvolinic acid and 7-desacetoxyhelvolic acid have been isolated from *Sarocladium oryzae* [102,103], but not yet from *A. fumigatus*. The fusidic acids may also be closely related, but has not been found in *A. fumigatus* [[104], pp. 264–265]. The reported toxicity of helvolic acid may be due to contamination with gliotoxin [1,23].

Helvolic acid was produced by nearly all strains (98%) we examined of *A. fumigatus* (Table 3).

*(10) Fumagillins (sesquiterpenes)*

*Trans*-fumagillin was isolated from *A. fumigatus* by Eble and Hanson [105], and its structure elucidated by Tarbbell [106,107] and McCorkindale and Sime [108]. Further extrolites in this biosynthetic family have been isolated later, inclusive fumagiringillin [109], demethoxyfumagillol [110], Sch528647 [111], RK-95113 [112] and closely related metabolites [113]. Ovalicin [114,115], FR-111142 [116], or FR65814 [117] may also be produced, but have been reported from other fungi. The fumitoxins, toxic to both animals and plants [118–122] were never structure elucidated, but based on the chemical data presented, they appear to be members of the fumagillin biosynthetic family. β-transbergamoten is a precursor of fumagillin [123].

We detected fumagillin in all strains of *A. fumigatus* examined (Table 3).

*(11) Fatty acids (fatty acids) and hexahydroxyprenyls (polyterpenes)*

*A fumigatus* has been reported to produce a series of hydroxypolyprenols [124], ubiquinones, phthioic acid and other lipids [125–127]. The role of these metabolites in the infection process is unknown, but other lipids (oxylipins) have been shown to be involved in virulence [128].

We did not screen for these lipids in the 40 extracts of *A. fumigatus*.

*(12) Siderochromes (N-hydroxyornithine with either three glycines or one glycine and two serins)*

Fusigen [129], ferricrocin and N′,N″,N‴-triacetylfusarinine C [130,132] are important iron-chelating extrolites from *A. fumigatus*, that may play a significant role in the infection process in animals [133,134].

As the production requires special substrates depleted for iron, we did not examine the cultures for the siderophores in our screening process.

*(13) Gliotoxins (phenylalanine, m-tyrosine, methionine)*

Gliotoxin was isolated from a strain of *A. fumigatus* by Johnson *et al.* [135] and Menzel *et al.* [98] and later structure elucidated [136]. Fumigacin [27,29] has been found in animal and human tissue, but fumigacin was later found to be a mixture of gliotoxin and helvolic acid. Gliotoxin has been claimed to be involved in diseases caused by *A. fumigatus* [40,137,138]. The less toxic bisdethiobis(methylthio)gliotoxin has also been reported from *A. fumigatus* [139,140] as has gliotoxin G, the tetrasulphide analogue of gliotoxin [139]. Other gliotoxins, including gliotoxin monoacetate [141,142], and gliotoxin E and G [143,144] may also be extrolites of *A. fumigatus*, but have been isolated from *Trichoderma virens* and *Penicillium lilacinoechinulatum* (Frisvad JC and Thrane U, unpublished).

Gliotoxin is best produced on media with low C/N ratio, so the media used here for screening of *A. fumigatus* extrolites were not optimal for its expression. When tested on such media [17] all isolates of *A. fumigatus* seems to be able to produce gliotoxin.

*(14) Fumigaclavins (tryptophane and terpene unit (dimethylallyl))*

Agroclavine, festuclavine, clymoclavine, chanoclavine I, fumigaclavine A, B, and C [15,145 149] are produced by *A. fumigatus*. The biosynthetic genes for the ergot alkaloids in *A. fumigatus* have been studied by Coyle and Panaccione [150]; Li and Unsöld [151]; Unsöld and Li [152], Stack *et al.* [153].

All 40 isolates examined of *A. fumigatus* produced fumigaclavine C (Table 3), the end-product in the biosynthetic family.

*(15) Fumitremorgirs, verruculogen, tryprostatins, cyclotrypostatins and spirotryprostatins (tryptophane, proline and terpene (dimethylallyl) groups)*

Brevianamide F [154,155] is a conceived precursor of the diverse biosynthetic family of fumitremorgins, including verruculogen [24,156–159], cyclotrypostatins [160], tryprostatins [161–163], spirotryprostatins [164,165], fumitremorgin A & B [140,166,172], fumitremorgin C [15,146,159,173], TR-2 [174], TR-3 =12,13-dihydroxyfumitremorgin C and demethoxyfumitremorgin C [162,163], 12,13-dihydrofumitremorgin C [159,175], and 15-acetoxyverruculogen [23]. In all, this extrolite family consists of 20 known members. Tryprostatin A is an inhibitor of microtubule assembly [176], and in general the fumitremorgins are cell cycle inhibitors and tremorgenic mycotoxins [23].

We found that the fumitremorgins (A, B, C), TR-2 and verruculogen were produced by all isolates of *A. fumigatus* examined (Table 3), but the full genome sequenced Af293 [31] only produce brevianamide F [17,177].

*(16) Simple diketopioperazines (two amino acids)*

Alanyl-leucyl and alanyl-isoleucyl, prolyl-phenylalanyl, prolyl-glycyl, prolyl-prolyl, prolyl-valyl, 4-hydroxyprolyl-leucyl, 4-hydroxyprolyl-phenylalanyl, and prolyl-leucyl diketopirazines, all consisting of L-amino acids, have all been reported from *A. fumigatus* [15,178,179]. Several of those are antibiotically active [15].

We did not detect any of those simple diketopiperazines in *A. fumigatus*.

*(17) Pyripyropenes (meroterpenoids and nicotinic acid)*

Pyripyropenes A-R have been reported from *A. fumigatus* [180–187]. The pyripyroperes have the ability to inhibit acyl-CoA:cholesterol acyltransferase and may thus play a role in the infection process.

We found that approximately half of the isolates of *A. fumigatus* produced pyripyropenes (48%, Table 3). We did not detect those metabolites in Af293, but the other full genome sequence strain of *A. fumigatus* (CBS 144.89 = CEA 10) did produced pyripyropenes.

*(18) Fumiquinazolines (anthranilic acid, tryptophane, valine)*

Fumiquinazolines A-E [140,188,189] were reported from marine isolates of *A. fumigatus*. These quinazolins have been reported to be moderately cytotoxic.

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We found that the fumiquinazolines were consistently produced by all 40 isolates of *A. fumigatus* examined (Table 3).

*(19) Tryptoquivalines (anthranilic acid, tryptophane, valine, terpene unit (dimethylallyl))*

The tryptoquivalines and tryptoquivalones were originally isolated from *A. clavatus* [190,191] but tryptoquivaline A and E to N has been reported from *A. fumigatus* [192–195]. It was later shown that *A. fumigatiaffinis* is a very efficient producer of (some of) these tryptoquivalines [11,14]. However, tryptoquivaline J was isolated from a strain of *A. fumigatus* by Afiyatullov [159], so it is possible that also *A. fumigatus* can produce at least some of these extrolites.

The tryptoquivalins were not detected in our analyses of 40 isolates of *A. fumigatus*. Earlier reports of tryptoquivalins [25] from *A. fumigatus* were apparently based on the fact that the UV-VIS spectra of the tryptoquivalins and the fumiquinazolines are quite similar. HPLC-HR-MS analysis showed that the major compounds with such UV spectra were all fumiquinazolines.

*(20) N-(2-cis(4-hydroxyphenyl)ethenyl)-formamide*

N-(2-cis(4-hydroxyphenyl)ethenyl)-formamide is a platelet aggregation inhibitor that was isolated from a strain identified as *A. fumigatus* [196].

We were not able to detect this extrolite in any of 40 extracts of *A. fumigatus*.

*(21) Restrictocins (polypeptides)*

Restrictocin, mitogillin and 'asp F1', small basic proteins, are cytotoxins that cleave ribosomal RNA [197–199]. The culture originally examined (ATCC 34475 =NRRL 2869) was first identified as *A. restrictus*, but later reidentified as *A. fumigatus* [200]. A leader sequence in the gene coding for these proteins protects the producer strains from suicide [201], and these proteins have also been identified as major allergens from the conidia, mycelium and culture filtrate of *A. fumigatus* [198]. 'Asp F1' was first found in urine of patients that suffered from invasive aspergillosis [197], so these compounds may be of significance in *A. fumigatus* mediated aspergillosis.

We did not screen *A. fumigatus* for any proteins in this study.

*(22) Volatile extrolites (including sesquiterpenes, alcohols and ketones)*

Sesquiterpenes provisionally detected from from *A. fumigatus* include 10(14)-(−)-aromadendrene, bicycloelemene, bicyclooctane-2-one, camphene, α-cadinene, 2-carene, caryophyllene, α & β-curcumene, cyclooctene, dihydroedulane I, β-elemol, α-farnesene, *trans*-β-farnesene, (−)-fenchol, germacrene A & B, italicene, a-longipenene, megastigma-4,6(E),8(Z)-triene, p-mentha-6,8-dien-2-ol, 2-methyl-2-bornane, 2-methylenebornanene, a-muurolene, neo-allo-ocimene, and β-phellandrene (39). Other volatile metabolites reported include 2-acetyl-5-methylfuran, anisole, 3-cycloheptane-1-one, 2,3-dimethylbutanoic acid methyl ester, 2,5-dimethylfuran, 4,4-dimethylpentenoic acid methyl ester, dodecane, 4-ethylbutan-4-olide, 2-ethylfuran, 2-ethyl-5-methylfuran, 1-ethyl-2-methylbenzene, furancol, 3-hexanone, isopropylfuran, 1-methoxy-3-methylbenzene, 2-methylbutanoic acid and its methyl ester, 4-methyl-2-(3-methyl-3-butenyl)furan, 3-methyl-1-heptene, 6-methyl-2-heptanone, 2-methyl-2,4-hexadiene, 2-methylphenole, 1-(3-methylpheryl)-ethanone, 3-octanone, 1,3,6-octatriene, styrene, 3,5,7-trimethyl-2E,4E,6E,8E-decatetraene, 2,3,5-trimethylfuran (39). The role of these volatiles in the infection process of *A. fumigatus*, if any, is unknown.

*(23) Primary metabolites*

The vitamin riboflavin has also been found in *A. fumigatus* [202,203], and so has several other primary widespread primary metabolites.

*(24) Biotransformations*

*A. fumigatus* is also capable of converting some plant metabolites for example melitolic acid to 4-hydroxycoumarin and o-coumaric acid to dicoumarol [204] and phenylacetic acid to 2,6-dihydroxyphenylacetic acid [205]. It is also not known whether this ability to bioconvert metabolites play a role in the animal infection process.

*(25) Proteins*

Apart from the restrictocins, *A. fumigatus* also produce hydrophobins and several extracellular enzymes and these do play a role in the fungal infection process [16,206].

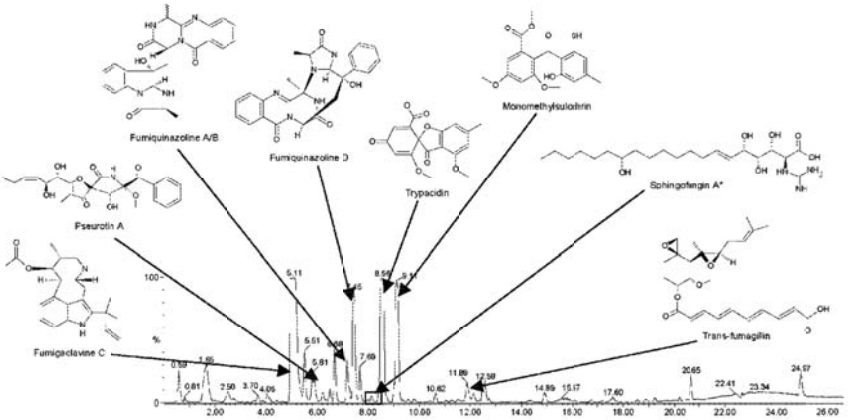


**Fig. 1** LC-MS BPI (base peak ion) ESI (electrospray ionization)+ trace of raw extract of *Aspergillus fumigatus* Af293 on CYA agar, depicting major and typical extrolites from the species. Box indicates window for the sphingofungins in this isolate. Sphingofungin A is one of four possible compounds with almost identical masses to be present, based on mass traces.

*(26) Extrolites erroneously reported from* Aspergillus fumigatus

*Aspergillus fumigatus* has been claimed to produce a large number of mycotoxins and other extrolites, including ochratoxin A [207 210], indications of aflatoxin [211], cyclopiazonic acid [212], kojic acid [213,214], sterigmatocystin [215] and fumifungin + viriditoxin [84]. The isolates producing these mycotoxins and other biologically active extrolites appear to be misidentified. For example cyclopiazonic acid is produced by *A. lentulus* and isolates of the latter species can have been mistaken for *A. fumigatus* [17]. The isolate reported to produce fumifungin also produced viriditoxin [84], and the latter is a typical metabolite produced by *A. viridinutans*, another member of *Aspergillus* subgenus *Fumigati* section *Fumigati* [14,216 218]. In the case of ochratoxin A, sterigmatocystin and aflatoxin, probably the mycotoxin itself was misidentified.

Molecules that may be artefacts, such as GERI-BP002-A [219] that is a sterol biosynthesis inhibitor, have been reported as extrolites of *A. fumigatus*. This compound may or may not be a real secondary metabolite.

Expansolide, antafumicin A & B, and cytochalasin E were all reported from a strain claimed to be *A. fumigatus* [220]. These extrolites have only been found in *A. clavatus* [221], so it is highly probable that the reported producing strain represented the latter species.

Ruakuric acid has been isolated from a strain of *A. fumigatus* growing in conjunction with a coral lichen in hot sulfurous springs, New Zealand [222]. We have not been able to examine this culture and we have not yet detected compunds with the characteristics of ruakuric acid from any strain of *A. fumigatus sensu stricto*.

Aurasperone C was reported from *A. fumigatus* by Mitchell *et al.* [36], but this metabolite is a common metabolite in *Aspergillus* section *Nigri* [223] and we have not been able to detect it in any strain of *A. fumigatus* in this study.

Fumigatonin was also reported from *A. fumigatus* [224], but the isolation of the chemically related novofumigatonin from *A. novofumigatus* [35] indicates that fumigatonin is produced only by *A. novofumigatus*.

None of the isolates of *A. fumigatus* examined here (Table 1) produced aflatoxins, antafumicins, cyclopiazonic acid, cytochalasin E, expansolides, kojic acid, ochratoxins, sterigmatocystin, or viriditoxin.

*(27) Extrolites of* A. fumigatus *Af293*

Af293, the full genome sequenced strain of *A. fumigatus* [31] produced, fumigaclavines, fumiquinazolines, trypacidin and mono-methylsulochrin, fumagillins,

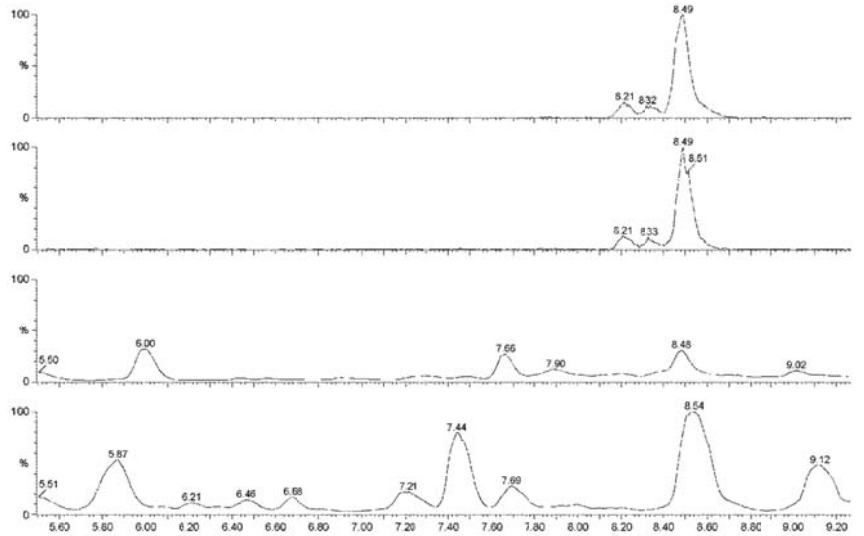


**Fig. 2** Comparison of *A. lentulus* and *A. fumigatus* production of sphingofungins. Though very different in general extrolite profile, both of these isolates produce the same profile of what appears to be sphingofungins. From the bottom: Af293 TIC (total ion count) ESI + (CYA agar); IBT 27201 *Aspergillus lentulus* TIC ESI + (CYA agar); 432.30±0.05 Da mass trace for Af293 and IBT 27201 respectively (this mass fits both sphingofungin A ($C_{21}H_{41}N_3O_6$) and sphingofungin C, D and fumifungin (the latter three: $C_{22}H_{41}NO_7$).

gliotoxins, pseurotins, chloroanthraquinones, fumitremorgins, verruculogen, helvolic acids and sphingofungins (Fig. 1). The presence of sphingofungins A, C, D, or fumifungin (or all of those) was indicated by HPLC-MS analysis (Fig. 2). The formulae of these extrolites and the other secondary metabolites produced by *A. fumigatus* are shown in Fig. 1 and Fig. 3.

## Discussion

We have been able to detect most of the major mycotoxins and other extrolites known from *A. fumigatus* in clinical strains, including the full genome sequenced Af293 and CBS 144.89 (=CEA10). These two strains produced gliotoxin, helvolic acid, fumagillin, fumigaclavine C, brevianamide F, fumiquinazolines and pseurotin A. Af293 and CBS 144.89 differed in that Af293 produced trypacidin, mono-methylsulochrin and some chloroanthraquinones, whereas CBS 144.89 produced a series of fumitremorgins (TR-2, fumitremorgin A, B, C and verruculogen). We also detected what we tentatively identified as sphingofungins in Af293, but have not screened other *A. fumigatus* strains for these extrolites yet. The other clinical strains also produced most of the well known secondary metabolites of *A. fumigatus* (Table 3), but 50% of the clinical strains did not produce fumigatin and pyripyropenes. However other investigators have found these extrolites in a lower proportion of the strains examined for example from silage [225] or from saw mills [226]. This may be because some of the strains were *A. lentulus* [17] or other strains similar to *A. fumigatus* [14]. Furthermore our results show that on the media CYA and YES only few strains show small peaks of gliotoxin, while they are produced in much higher amounts on media such as yeast extract agar (YE) [17]. Therefore the frequency of gliotoxin (38%) in *A. fumigatus* may be much higher in reality. On the other hand, the media CYA and YES are very good media for production of most other secondary metabolites from *A. fumigatus*, and we recommend to use those media in screening for all other secondary metabolites than gliotoxin.

The number of biosynthetic families of secondary metabolites reported to be produced by *A. fumigatus*

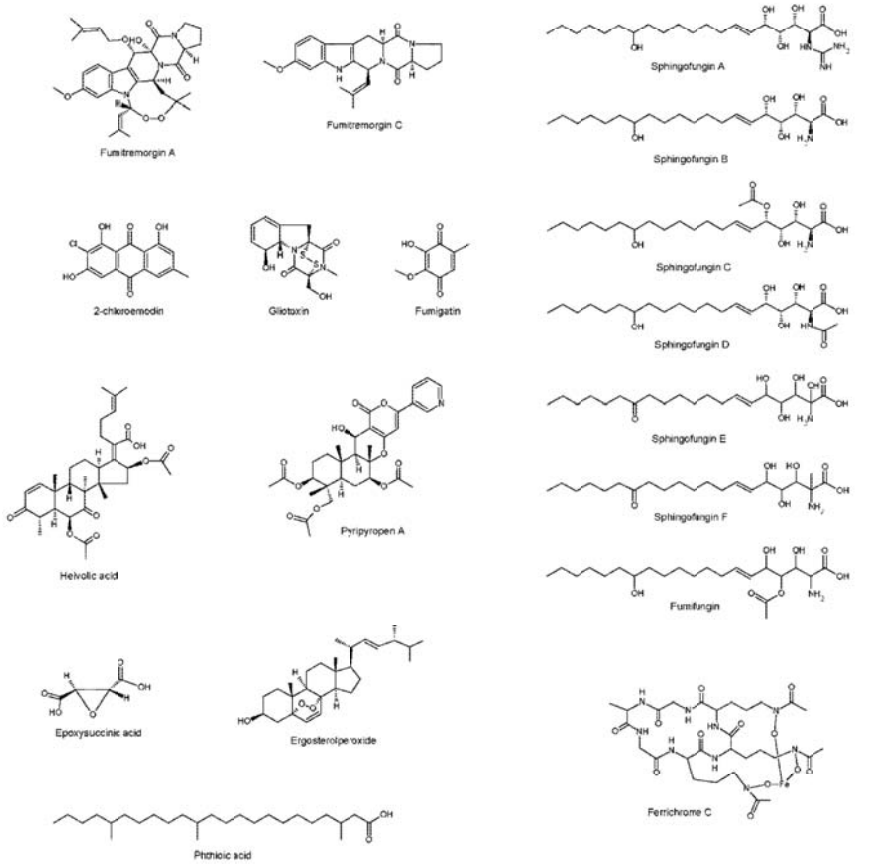


**Fig. 3** Collection of single extrolites presenting the different compound classes, in addition to those in Fig. 2, produced by *Aspergillus fumigatus*.

(24) is impressive and so is the number of individual extrolites [226] (Table 2). We have not screened for all these 226 metabolites, but have concentrated on the most toxic or otherwise bioactive major metabolites from each biosynthetic family. It is still an open question how many of these secondary metabolites are involved in the infection process of lungs, but there is evidence that at least gliotoxin is involved [21,40,43, 137,138,227].

Nierman *et al.* [31] found by bioinformatic analysis of the full genome of *A. fumigatus* that 14 gene clusters indicated that they coded for non ribosomal peptide synthases (NRPS). Examination of the chemical phenotype (exometabolome) of *A. fumigatus* shows that gliotoxins, fumigaclavines, fumitremorgins, fumiquinazolins, siderochromes, diketopiperazines, restrictocins, N-(2-cis(4-hydroxyphenyl)ethenyl-formamide, and possibly tryptoquivalnes, in addition to amino acids that

**Table 2** Extrolites produced by isolates of *Aspergillus fumigatus*

| Extrolite family | Number of members | Individual members |
|---|---|---|
| 1. Epoxysuccinic acid | 1 | Epoxysuccinic acid |
| 2. Fumigatins | 21 | Fumigatin<br>Fumigatin oxide<br>Fumigatin chlorohydrins<br>Fumigatol<br>Spinulosin<br>Spinulosin hydrate<br>Spinulosin quinol-hydrat<br>Dihydrospinulosin quino<br>Phyllostine<br>Orsellinic acid<br>Orcinol<br>m-cresol<br>3,4-dihydroxytoluquinone<br>4-hydroxy-3-methoxytoluquinone<br>3-hydroxytoluquinone<br>3,6-dihydroxytoluquinone<br>3-hydroxy-4-methoxy-<br>Toluquinone 1,6-epoxide<br>4-carboxy-5,5-dihydroxy-3,3′-dimethyldiphenyl<br>Fumiquinone A<br>Fumiquinone B |
| 3. Trypacidins | 6 | Trypacidin<br>Bisdechlorogeodin<br>Monomethylsalochrin<br>Sulochrin-2′-methylether<br>Asperfumin<br>Asperfumoid |
| 4. Anthraquinones and anthrones | 5 | Emodin<br>Physcion<br>2-chloroemodin<br>2-chloro-1,3,8-trihydroxy-<br>6-methylanthrone<br>2-chloro-1,3,8-trihydroxy-<br>6-hydroxymethylanthrone |
| 5. Melanins | 8 | YWA1<br>1,3,6,8-THN<br>Flaviolin<br>Scytalone<br>1,3,8-THN<br>2-HJ<br>Vermelone<br>1,8-DHN |
| 6. Sphingofungins | 7 | Sphingofungin A-D<br>Sphingofungins E-F?<br>Fumifungin |
| 7. Pseurotins | 11 | Pseurotin A-E<br>8-O-demethylpseurotin A<br>Synerazol<br>RK-95113<br>Azaspirene?<br>FD-839<br>Pseurotin F2 |
| 8 Sterols | 7 | Ergosterol<br>Ergosterolperoxide<br>Ergosterolpalmitat<br>24-methylenophenol<br>Ergosta-4,6,8(14),22-tetraen-3-one<br>Ergosta-4,22-dicne-3β-ol 5α,8α-Epidioxy-ergosta-<br>6,22-dicne-3β-ol |
| 9. Helvolic acid | 3 | Helvolic acid<br>Helvolinic acid<br>7-desacetoxyhelvolic acid |
| 10. Fumagillins | 13 | Fumagillin<br>Fumitoxins A-D<br>Fumagillol |

**Table 2** (*Continued*)

| Extrolite family | Number of members | Individual members |
|---|---|---|
| | | 5-Demethoxyfumagillol |
| | | Fumagiringillin |
| | | FR-111142 |
| | | Sch528647 |
| | | RK-95113 |
| | | Ovalicin? |
| | | β-*trans*-bergamotene |
| 11. Hexahydropolyprenyls | 5 | Hexahydropolyprenyls A-E |
| 11a. Phthioic acid | 2 | Phthioic acid |
| | | Teichoic acid |
| 11b. Hydroxyphenyls | 1 | bis(3-hydroxy-3-tert. butyl-5-Methylphenyl)methane (= GER1-BP002-A) |
| 12. Siderochromes | 2 | Fusigen |
| | | Ferrichrome |
| 13. Gliotoxins | 5 | Gliotoxin |
| | | Gliotoxin F |
| | | Gliotoxin G |
| | | S-methylgliotoxin |
| | | bisdethiobis(methylthio)-Gliotoxin |
| 14. Fumigaclavins | 7 | Agroclavine |
| | | Elymoclavin |
| | | Chanoclavine I |
| | | Festuclavin |
| | | Fumigaclavine A-C |
| 15. Fumitremorgins | 20 | Brevianamide F |
| | | Fumitremorgin A-C |
| | | Verruculogen |
| | | 15-acetoxyverruculogen |
| | | Demethoxyfumitremorgin C |
| | | 12,13-dihydroxyfumitremorgin C (= TR-3) |
| | | 12,13-dihydrofumitremorgin C |
| | | TR-2 |
| | | Cydotryprostatin A-D |
| | | Dehydrotryprostatin |
| | | Tryprostatin A & B |
| | | Spiotryprostatin A & B |
| | | 'Compound 6' |
| 16 Simple diketopiperazines | 9 | Alanyl-leucyl and alanyl-isoleucyl, prolyl-phenylalanyl, prolyl-glycyl, prolyl-prolyl, prolyl-valyl, 4-hydroxyprolyl-leucyl, 4-hydroxyprolyl-phenylalanyl, and prolyl-leucyl diketopirazines |
| 17. Pyripyropenes | 19 | Pyripyropene A-R |
| | | GER1-BP001-A |
| 18. Fumiquinazolines | 7 | Fumiquinazoline A-G |
| 19. Tryptoquivalins | 11 | Tryptoquivaline? |
| | | Notryptoquivaline? |
| | | Tryptoquivaline E-N? |
| 20. N-(2-cis(4-hydroxyphenyl)ethenyl-formamide 1 | | |
| 21. Restrictocins | 3 | Restrictocin |
| | | Mitogillin |
| | | Asp F1 |
| 22. Volatile extrolites | 25–28 | |
| Sum: | | |
| 24 Biosynthetic families | 226 | secondary metabolites |

involve the addition of the polyketides: sphingofungins, pseurotins, trypacidins, pyripyropenes are all NRPS dependent. This could account for in all, 13 biosynthetic families that involve amino acids. However, many of the secondary metabolites of *A. fumigatus* are of mixed biosynthetic origin. Concerning terpene involvement helvolic acids, ergosterolperoxide, fumagillin, hexahydropolyprenols are pure terpene secondary metabolites, but the fumigaclavines, fumitremorgins and tryptoquivalines have added dimethylallyl groups, so at least 7 gene clusters should contain genes coding for terpene biosynthesis. Nierman *et al.* [31] found that there were 7

**Table 3** Consistency in production of extrolites by *Aspergillus fumigatus* from different sources as evaluated using HPLC and isolates grown on CYA and YES agar for one week at 25°C

| Isolate | Source | Secondary metabolite family[f] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 3 | 4 | 9 | 10 | 13 | 14 | 15 | 18 | 7 | 17 |
| CBS 542.75 | man, USA | − | − | − | + | + | + | + | + | + | + | + |
| CBS 143.89 | man, F | − | − | − | + | + | − | + | + | + | + | + |
| CBS 144.89 | man, F | | | | | | | | | | | |
| CBS 145.89 | man, F | − | − | − | + | + | − | + | + | + | + | − |
| CBS 146.89 | man, F | | | | | | | | | | | |
| CBS 147.89 | man, F | | | | | | | | | | | |
| IBT Stend1 | man, DK | − | + | − | + | + | − | + | + | + | + | − |
| IBT Stend2 | man, DK | + | + | − | + | + | − | + | + | + | + | − |
| Af293 | man, UK | − | + | − | + | + | + | + | + | + | + | − |
| CBS 133.61[f] | Chicken lung | − | + | | + | − | + | + | + | + | + | − |
| IBT 16904 | Saltern,SL | + | + | − | + | + | − | + | + | + | + | + |
| IBT 16902 | Saltern, SL | + | + | − | + | + | + | + | + | + | + | + |
| IBT 16903 | Saltern, SL | + | + | − | + | | − | + | + | + | + | + |
| IBT 16901 | Saltern, SL | + | + | − | + | + | − | + | + | + | + | + |
| IBT 24004 | Saltern, SL | − | + | − | + | + | − | + | + | + | − | + |
| ATCC 32722 | Soil, CAN | | | · | | | | | | | | |
| NRRL 1979 | Soil, USA | + | + | − | + | + | +* | + | + | + | + | + |
| CBS 113.26 | Soil, D | | | · | | | | | | | | |
| CBS 132.54 | Soil? | | | | | | | | | | | |
| CBS 457.75 | Soil, IND | | | | | | | | | | | |
| CBS 151.89 | Stone, D | | | · | | | | | | | | |
| CBS 152.89 | stone, D | + | + | − | + | + | − | + | + | + | + | + |
| NRRL 5587 | ? | − | − | − | + | + | − | + | + | + | + | − |
| IBT D47I | soil, INDO | − | + | − | + | + | − | + | + | + | + | − |
| CCRC 32120 | soil, TAI | − | + | − | + | + | − | + | + | + | + | − |
| IBT 25732 | soil, K | − | + | − | + | + | − | + | + | + | + | − |
| LMI 376380 | ? | − | + | − | + | − | − | + | + | + | + | + |
| CBS 545.65 | ? | − | + | − | + | − | − | + | − | + | + | − |
| WB 5033 | ? | − | + | − | + | + | + | + | + | + | + | − |
| IBT 23737 | ?, DK | + | + | − | + | + | − | + | + | + | + | − |
| ATCC 32722 | ?, USA | + | + | − | + | + | + | + | + | + | + | − |
| CBS 192.65 | Feed, NL | + | + | − | + | + | + | + | + | + | + | + |
| CBS 148.89 | Maize, F | | | · | | | | | | | | |
| CBS 149.89 | Maize, F | | | | | | | | | | | |
| CBS 150.89 | Beetroot, F | | | · | | | | | | | | |
| IBT 21997 | feed, E | | | · | | | | | | | | |
| IBT 22023 | silage, D | − | + | − | + | + | + | + | + | + | + | − |
| IBT PerHag | Silage, S | − | + | − | + | + | + | + | + | + | + | − |
| IBT 22234 | Tea factory, U | + | + | − | + | + | − | + | + | + | + | − |
| IBT | food, I | + | + | − | + | + | − | + | + | + | + | − |

[f] Metabolite family nr (frequency of extrolite production out of 40 strains).
*Gliotoxin detected originally.
2. Fumigatin (35%).
3. Trypacidin (75%).
4. Chloro-anthraquinones or -anthrones (70%).
9. Helvolic acid (98%).
10. Fumagillins (93%).
13. Gliotoxin (>38%).
14. Fumigaclavines (100%).
15. Fumitremorgins (98%).
18. Fumiquinazolines (100%).
7. Pseurotins (100%).
17. Pyripyropenes (48%).

gene clusters accounting for dimethylallyl tryptophane synthases and here we can account for three of these. However pure terpene secondary metabolite clusters were not mentioned. It has been shown that the fumonisins are not only depending on the fumonisin gene cluster, but also on regulating genes on other chromosomes and environmental factors [228,229]. Finally polyketide synthases (PKS) include fumigatin, trypacidins, chloroanthraquinones, melanins, sphingofungins, pyripyropenes and pseurotins, accounting for at least 7 PKS gene clusters, only half of the 14 PKS gene clusters listed by Nierman *et al.* [31]. However, the PKS and NRPS gene clusters may be differently organized and thus pure PKS gene clusters and mixed PKS-NRPS clusters may be difficult to detect using bioinformatic methods. The gene clusters for gliotoxin, fumitremorgins, fumigaclavines and siderophores have been provisionally detected [153,177,227,230].

The production of sphingofungins by *A. fumigatus* has been reported previously in *A. fumigatus* ATCC 20857 [81,82], and the closely related sphingofungin called fumifungin was isolated from a fungus identified as *A. fumigatus* Y-83,0405 [84]. We have not been able to get those cultures, but the latter appear to be *A. viridinutans* rather than *A. fumigatus*, as it also produced viridiotoxin. The detection of compounds with the mass around 432.3 strongly indicates that *A. fumigatus* Af 293 and *A. lentulus* IBT 27201 produce these sphingofungins, but as sphingofungin A, C, D or fumifungin almost have the same mass, it would be necessary to isolate, purify and characterize these compounds using NMR to be sure they are actually sphingofungins. It is intriguing that the mass trace for *A. fumigatus* for these compounds is almost identical to that of *A. lentulus*, indicating a common biosynthesis. Since there are few secondary metabolites in common between *A. fumigatus* and *A. lentulus* [17], it is tempting to speculate that the sphingofungins might be involved in the lung infection process. All 10 species of *Aspergillus* and 23 species of *Neosartorya* should be analyzed for sphingofungins to see if all or only the pathogenic species produce them. The production of sphingofungins was consistent on YES and CYA agar only with variations in amounts.

We have shown that strains of *A. fumigatus* produce the same profile of secondary metabolites, but that all metabolites are not necessarily expressed phenotypically in any given strain. The new possibilities of bioinformatic search based on full genome sequenced strains will show whether genes for fumigatin and for pyripyropenes are present in a strain like Af293, where we as yet have never detected these secondary metabolites. If the geneclusters are present, they may be silent or defective in the strains that do not produce them. However, many strains consistently produce a series of different secondary metabolites, the most consistent being fumigaclavines, fumitremorgins, fumiquinazolins, pseurotins, helvolic acid and fumagillin. The polyketides are less consistently expressed, but maybe other growth media or stimuli will induce their production.

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# Paper 5

## Distribution of sterigmatocystin in filamentous fungi

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# Distribution of sterigmatocystin in filamentous fungi

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**Abstract**

The carcinogenic sterigmatocystin has over the last five decades been reported from several different genera, sometimes together with the biosynthetically related and more toxic aflatoxins: *Aschersonia*, *Aspergillus*, *Bipolaris*, *Chaetomium*, *Emericella*, *Eurotium*, *Farrowia*, *Fusarium*, *Humicola* and *Moelleriella*, and *Monocillium*, including the economically important species *Aspergillus flavus* and *A. parasiticus* and the model organism *Emericella nidulans*. We have reviewed the reported producers and reevaluated the important metabolites in the aflatoxin biosynthesis from a representative number of available isolates. The fungi were grown on a selection of secondary metabolite inducing media and analyzed with HPLC-UV/Vis diode array detection, – HRMS and – MS/MS. Along with a clarification of previous findings, one new producer of aflatoxin was found: *Aspergillus togoensis* (formerly *Stilbothamnium togoense*). The wide distribution of the large sterigmatocystin and aflatoxin gene clusters was investigated with using DNA sequencing and the results shows how several completely unrelated fungi can produce the same secondary metabolites. Sterigmatocystin was confirmed in *Emericella acristata*, *E. astellata, E. aurantiobrunnea, E. bicolor, E. cleistominuta, E. dentata, E. discophora, E. echinulata, E. falconensis, E. foeniculicola, E. foveolata, E. fructiculosa, E. heterothallica, E. lata, E. navahoensis, E. nidulans, E. olivicola, E. parvathecia, E. rugulosa, E. stella-maris, E. striata, E. venezuelensis*, *Aspergillus versicolor*, *A. ochraceoroseus*, *A. rambellii*, *A. togoensis*, *Chaetomium cellulolyticum, C. longicolleum, C. malaysiense, C. udagawae, C. virescens* and *Humicola fuscoatra* and found for the first time in *A. asperescens*, *A. aureolatus*, *A. eburneocremeus*, *A. protuberus*, and *Penicillium inflatum*. Based on biosynthetic reasoning all aflatoxin producers in *Aspergillus* section *Flavi* must have the capability to produce sterigmatocystin, but we did not detect it in any of those species. Rather than being accumulated, sterigmatocystin is rapidly converted into O-methylsterigmatocystin and aflatoxins in section *Flavi*. The immediate precursor for aflatoxin, O-methylsterigmatocystin was found in *Chaetomium cellulolyticum, C. longicolleum, C. malaysiense* and *C. virescens*, but aflatoxins were not detected in any *Chaetomium* species.

**Introduction**

Sterigmatocystin is a carcinogenic polyketide produced by several different fungi, spanning many genera. The toxic metabolite is coupled to the more potent aflatoxins as a precursor in aflatoxigenic strains. Aflatoxins are perhaps the most extensively studied fungal extrolites, both in terms of biological function and biosynthetic understanding. Fungi capable of producing sterigmatocystins and aflatoxins are common food contaminants, and plant and mammalian pathogens, and thus pose an important safety issue and have a large economic impact on many biotechnological industries.

The chemical analyses and screenings of aflatoxin and inherently sterigmatocystin spans five decades, leaving many publications/discoveries to be re-evaluated and scrutinized using modern methods and instruments.

Complex secondary metabolites or extrolites are specialized structures developed through numerous evolutionary iterations against biological challenges. However, most of them cannot be used as chemotaxonomical markers, since the same metabolite can occur in different species within one genus. It is on the other hand less common that they are observed in more than one genus. Sterigmatocystins/aflatoxins are distinct metabolites that do not follow this rule: so far sterigmatocystins has been reported from many species in several different genera: *Aspergillus* (and some of its perfect states such as *Petromyces, Chaetosartorya, Emericella*, and *Eurotium*), *Bipolaris (Drechslera)*, *Chaetomium*, *Farrowia*, *Fusarium*, *Humicola, Monocillium, Moelleriella* and *Penicillium*. The presence of such complicated natural products in phylogenetically widely different fungi is very interesting given the complex gene cluster coding for sterigmatocystins / aflatoxins, and we wanted to find out which fungi could actually produce these mycotoxins.

**Sterigmatocystin and aflatoxin**

Aflatoxin and sterigmatocystin research dates back to the middle of the last century, with the Turkey X-disease (Blount, 1961) as a catalyst, for a rapidly expanding knowledgebase. Sterigmatocystin was known prior to the aflatoxins and first mentioned in 1948 by Birkinshaw and Hammady (Birkinshaw and Hammady, 1957) and Nekam and Polgar (Nekam and Polgar, 1948) where it was partially purified from a *Sterigmatocystis* (= *Aspergillus*) sp. as a *Staphylococcus* inhibiting agent. In 1954 Hasuda et al. (Hatsuda et al., 1954; Hatsuda and Kuyama, 1954) succeeded in isolating the metabolite from *Aspergillus versicolor*. The relative structure was correctly established in 1962 (Bullock et al., 1962). In 1968 Holker et al. (Holker and Mulheirn, 1968) showed that the degradation product (S)-2-methylbutanoic acid of sterigmatocystin was the same as had been shown for aflatoxin B and G's, which then had their absolute stereo chemistry determined (Brechbühler et al., 1967). Fukuyama et al. later used crystallography to prove the absolute stereochemistry of sterigmatocystin and O-methylsterigmatocystin (Fukuyama et
al., 1976; Fukuyama et al., 1975). The correlation in absolute stereochemistry of sterigmatocystin and aflatoxin indicates the shared biosynthetic route; sterigmatocystin is the second to last step known in

the biosynthesis of aflatoxin with O-methylsterigmatocystin as the final intermediate. The entire biosynthesis of sterigmatocystin involves 15 steps (Yabe and Nakajima, 2004). O-methylsterigmatocystin is normally not seen in non-aflatoxic species, except *Chaetomium* spp. (*C. malaysiense* and *C. longicolleum*) and is therefore a good indicator for a potential aflatoxin producing isolate. A taxonomic overview of aflatoxin producers have been given by Varga et al. (Varga et al., 2009).

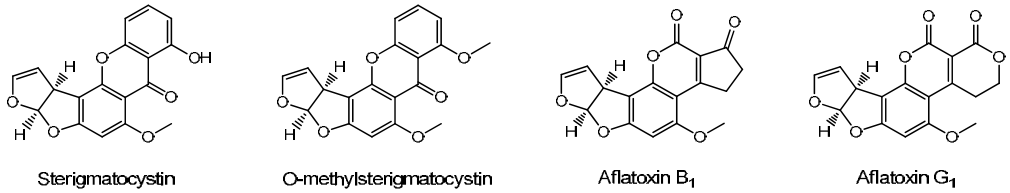


**Figure 1.** The important products of the sterigmatocystin/aflatoxin pathway: sterigmatocystin, O-methylsterigmatocystin, aflatoxin $B_1$ and $G_1$.

**Biological activity**

One of the earliest reports that can be linked to sterigmatocystin is the report by Nekam and Polgar, that a non-isolated compound (named sterigmatocystin) in colonies of *Sterigmatocystis* sp. showed an antibiotic behavior against *Staphylococcus aureus* (as *Micrococcus pyogenes* var. *aureus*) (Nekam and Polgar, 1948), but Davies et al. later reported no antibacterial or anti amoebae activity of sterigmatocystin (Davies et al., 1960). It appears that sterigmatocystin is not an effective antibiotic.

The early linkage of sterigmatocystin to aflatoxin also included bioactivity correlation, given the presence of the active moiety of the bisfuran ring in both mycotoxins and one of the first in vivo studies by Dickens et al. did indeed reveal its hepacarcinogenic potential (Dickens et al., 1966) and this was later confirmed in several studies (Holzapfel et al., 1966; Purchase and van der Watt, 1968; Purchase and van der Watt, 1970). The metabolites couples to DNA via an epoxidation of the bisfuran ring and succesquently covalent bonding to guanine residues of DNA (Essigmann et al., 1977; Harris et al., 1989; Bedard and Massey, 2006).

These results were confirmed and expanded to include a variety of fungi and other microorganisms (Lillehoj and Ciegler, 1968; Bradner et al., 1975; Banu and Shanmugasundaram, 1985). Bradner et al. tested sterigmatocystin, 5-methoxysterigmatocystin and other derivaties for antitumor activity and found that especially 5-methoxysterigmatocystin increased survival rate of mice with leukemia (Bradner

et al., 1975). Compared to aflatoxin $B_1$ (the most potent of the aflatoxins), sterigmatocystin is approximately 150 times less carcinogenic when administrated orally, but often occurring in much

higher amounts than the aflatoxins. Therefore, sterigmatocystin and analogues are very important mycotoxins for human and animals (Terao, 1983).

Sterigmatocystin (and aflatoxin $B_1$) has more recently been found to induce apoptosis in human peripheral blood lymphocytes, thus compromising the immune systems alongside its carcinogenic effects (Sun et al., 2002). Sterigmatocystin has shown a much higher toxicity for a lung cell line than liver cells (Bunger et al., 2004), a noticeably result given the high concentrations of aflatoxin, and thereby inherently also sterigmatocystin, that farmers can be subjected to when inhaling aflatoxin-contaminated dust (Selim et al., 1998). Hepatitis B virus has been shown to work in synergy with aflatoxin, resulting in a decreased nuclear extention repair of aflatoxin-DNA adducts (Kew, 2003).

Interestingly, the ecological function of sterigmatocystin has never been fully clarified, but the ecological function of sterigmatocystin and aflatoxin is likely to be insectidal against fungivorous insects in synergy with other toxic natural products for protection of conidia and sclerotia (Matsumura and Knight, 1967; Wicklow and Cole, 1982; Gloer et al., 1988; Ohtomo et al., 1975).

**Sterigmatocystin and aflatoxin producing strains**

Contrary to most other complex secondary metabolites, sterigmatocystin is found in several different genera. As mentioned earlier, it was first associated with a *Sterigmatocytis* sp. (synonym of *Aspergillus*). *Aspergillus* section *Flavi* has the most dominant producers of sterigmatocystin and especially aflatoxin: *A. flavus* and *A. parasiticus*, which are important species in especially maize and peanut contaminations and *A. minisclerotigenes* and *A. parvisclerotigenus* found in warmer climates and less often as agricultural contaminants, but they display a much more vigorous and constant aflatoxin production on artificial media compared to *A. flavus* (Egel et al., 1994; Pildain et al., 2008). The *Stilbothamnium togoense* is a synonym of *Aspergillus togoensis* and produce sclerotia and synnemata. Roquebert and Nicot suggested that the species is similar to “an ancestral form” of *Aspergillus* section *Flavi* (Roquebert and Nicot, 1985). McAlpin who found a mutant (NRRL 29254) of *A. flavus* (NRRL 3357) to produce synnemata and stipitate sclerotia on selected media, thus supporting that *S. stilbothamnium* is in *Aspergillus* sp. (Mcalpin, 2001). Wicklow et al. found *A. togoense* to produce large amounts of sterigmatocystin when grown on steam-sterilized rice, but did not detect any aflatoxin nor cyclopiazonic acid, both important metabolites of *A. flavus* (Wicklow et al., 1989).

More recently *Aspergillus* have been expanded with section *Ochraceorosei*, which includes the two closely related aflatoxigenic species *A. ochraceoroseus* and *A. rambellii* and section *Ochraceurosei* was regarded as being closer to section *Nidulantes* than to section *Flavi* (Frisvad et al., 2005). The *Aspergillus*

subgenus *Nidulantes* section *Nidulantes* and section *Versicolores* both contain a large number of sterigmatocystin producing species (Cole and Cox, 1981). The model organism *Emericella nidulans*

can produce large amounts of the mycotoxin (Frisvad, 1985; Hajjar et al., 1989; Barnes et al., 1994; Klich et al., 2001).

The aflatoxin gene cluster of the *Aspergillus ochraceoroseus*, *E. nidulans* and *A. rambellii* was analyzed in depth by Cary et al. (2009) and they concluded that *A. ochraceoroseus* and *A. rambellii* are closely related and their gene clusters have identical organization, supporting the rDNA analysis of (Frisvad et al., 2005). Interestingly the *aflQ* and *aflP* genes responsible for conversion of sterigmatocystin to aflatoxin in *A. flavus* and *A. parasiticus* were not found inside the aflatoxin gene cluster of *A. ochraceoroseus* and remains to be identified among possible candidates. Homologue sequences were found several places in the available *E. nidulans* genome.

Recently Schmidt-Heydt et al. (Schmidt-Heydt et al., 2009) reported the first *Fusarium* (*F. kyushuense* (NRRL 3509) to produce aflatoxin $B_1$ and $G_1$. Even though highly selective methods were used to detect the aflatoxin, it is still so controversial that it requires verification of another lab. In the analysis of *F. kyushuense* Schmidt-Heydt et al. found the *nor*-1 gene to have >90% sequence similarity to that of *A. flavus*. Interanalysis with sterigmatocystin and aflatoxin gene clusters from different species, will hopefully reveal how the *Fusarium* aflatoxin gene cluster is organised and what similarities can be found in other *Fusarium* species.

**Structurally related compounds**

Anthraquinones and xanthones (see Figure 2) are very common metabolites to many filamentous fungi and as Cary and Ehrlich hypothesized, the aflatoxin/sterigmatocystin gene cluster could be a functional development from a common anthraquinone genecluster established for light protection of conidial spores (Cary and Ehrlich, 2006).

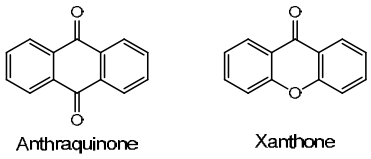


**Figure 2.** Basic structure of the important polyketides antraquinone and xanthone.

The structural significant motif of sterigmatocystin and aflatoxin (and the common precursor versicolorins) is the bisfuran moiety, which also holds the key to the bioactivity in animal cell studies. The feature is quite unique among known microbial products (Laatsch, 2009) and very few analogues exists: austocystins from *A. ustus* (section *Nidulantes*) (Steyn and Vleggaar, 1974; Steyn and Vleggaar, 1975) and dothistromins, are both closely related to versicolorins (anthraquinones) (see Figure 3). Doshistromins are produced by *Dothistroma septosporum*, a pine tree pathogen that causes severe

damage to New Zealand pine forests (Bear et al., 1972; Gibson, 1972). Dothiostromins are also produced by other members of ascomycetes Dothideales, particularly several members of the *Cercospora* species (Assante et al., 1977; Stoessl, 1984). Sequencing of the dothistromin gene cluster have revealed a large homology with the aflatoxin biosynthetic pathway genes, possibly presenting an ancestral type of the gene cluster, though the *D. septosporum* cluster is dived into three mini clusters (Bradshaw et al., 2002; Bradshaw et al., 2006; Bradshaw and Zhang, 2006; Cary and Ehrlich, 2006; Zhang et al., 2007).

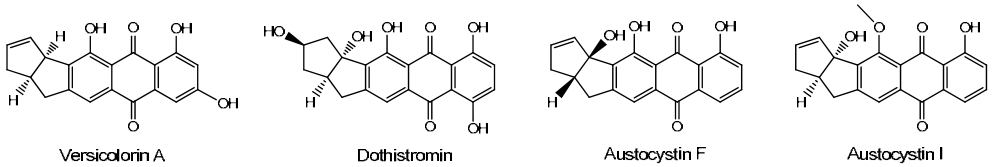


**Figure 3.** The sterigmatocystin presusors versicolorin A and structurally related compounds: dothiostromin (*Dothistroma septosporum* and *Cerospora* sp.) and austocystins (*A. ustus*).

**Growth conditions**

Growth conditions and especially nutrients have a significant impact on the production of sterigmatocystin and aflatoxin. The many different genera – and species even – require different media to produce the toxins in highest yields: media that for one culture results in optimal sterigmatocystin production can fail completely for other fungi, such that the metabolite is untraceable (Barnes et al., 1994). The nutritional regulation of sterigmatocystin and especially aflatoxin production generally require simple sugars, low pH, a reduced nitrogen source and mild oxidative stress, as reviewed for aflatoxin (in *Aspergillus*) by Georgianna and Payne (2009). The use of yeast extract agar was investigated for *Aspergillus parasiticus* by Williams et al. (Wilkinson et al., 2007) and both yeast extract (YE) and a sucrose enriched YE (YES) induced aflatoxin production. The more distant *Dothistroma pini* fungi has also been found to produce high amounts of dothistromin on yeast and malt extract containing media as well as on malt extract agar (Bradshaw et al., 2000).

Our experience with growth conditions, especially nutritional requirements, confirms the high degree of selectivity in metabolite production. YES agar is predominantely the best selection for high aflatoxin
production in *Aspergillus* and *Emericella* (Frisvad and Samson, 2004; Frisvad et al., 2005; Frisvad et al., 2004), but for *Chaetomium* (and occasionally *Emericella*) OAT agar facilitates a higher sterigmatocystin production. For all three genera, WATM is a good alternative that can deliver a high sterigmatocystin production. An incubation period of 7 days at 25°C in dark is sufficient for good producers to give significant levels of sterigmatocystin (and aflatoxin), but some isolates require 14

or 20 days to reach a detectable level. All three media are complex media with many less well "defined" ingredients that may themselves be acting regulatory on the cultivated fungi on a epigenetic level, as many of these epigenetic modifiers have been found so far to be small molecules with resemblance to nucleotides, fatty acids and other simple signal metabolites (Henrikson et al., 2009; Williams et al., 2008). Studies on the effect of various fatty acis in aflatoxin prooduction have shown the potential for a much increased output (Tiwari et al., 1986).

**Habitats**

The samples analysed have been collected in many different habitats as well as many of the species are cosmopolitan fungi, found around the world, however many of the species are predominantly isolated from soil, crop fields, nuts, figs and insects which might correlate to the insectide linkage to aflatoxins (and sterigmatocystins) (Wicklow and Cole, 1982; Gloer et al., 1988). It has been shown that sterigmatocystin's coupling to sporulation, might improve fungal fitness for survival (Wilkinson et al., 2004). This hypothesis can be extended to the longitudes, from where the isolates have been sampled and most of the potent aflatoxin producers are predominantly found in tropical or sub-tropical climates: *A. minisclerotigenes*, *A. parvisclerotigenus*, *A. parasiticus*, *A. nomius*, *A. Arachidicola* and *A. bombysis* and now also *A. togenose.* The other newly proposed aflatoxin producer *F. kyushuense*, only exists in very few isolates and knowledge about the general distribution is limited.

**Incorrect assignments**

The discovery of sterigmatocystin in the 1950$^{th}$ and aflatoxin in the 1960$^{th}$ and their biological and economical significance has resulted in numerous different studies, with improving instrumental methods during the years. Many results are, however, based on thin-layer chromatography (the reason aflatoxins are named $B_{1,2}$,$G_{1,2}$, are the color in UV-light – blue and green – and their relative position on the TLC plates). Fungi are capable of producing many UV- fluorescing compounds, such as indole-containing metabolites of tryptophane origin, and these can give erroneous results using TLC. Contaminated cultures have also resulted in several incorrect assignments, especially *Aspergillus flavus*/*A. oryzae* and *A. parasiticus*/*A. sojae*, which respectively can be challenging to separate morphologically, have generated incorrect reports. A more thoroughly review of the incorrect assignments can be found in Varga et al. (2009).

## Results and Discussion

Sterigmatocystin production was verified in the following species: *Emericella acristata*, *E. astellata, E. aurantiobrunnea, E. bicolor, E. cleistominuta, E. dentata, E. discophora, E. echinulata, E. falconensis, E. foeniculicola, E. foveolata, E. fructiculosa, E. heterothallica, E. lata, E. navahoensis, E. nidulans, E. olivicola, E. parvathecia, E. rugulosa, E. stella-maris, E. striata, E. venezuelensis*, *Aspergillus versicolor*, *A. ochraceoroseus*, *A. rambellii*, *A. togoensis*, *Chaetomium cellulolyticum, C. longicolleum, C.*

*malaysiense, C. udagawae, C. virescens* and *Humicola fuscoatra* (Table 1). It was further for first time found in *A. asperescens*, *A. aureolatus*, *A. eburneocremeus* and *A. protuberus*. More surprisingly is it was found in *Penicillium inflatum* described by Stolk and Malla (1971). They placed this species in *Penicillium* because it had no definite foot-cell, the metulae did not develop simultaneoulsy and the conidiophore wall was thin, strongly indicating a *Penicillium* species. This is the first confirmed report of a *Penicillium* producing sterigmatocystin. However, a species described as *Aspergillus tardus* (Bissett and Widden, 1984) was placed in *Aspergillus* section *Versicolores* by Klich (1993), but also appears to be closely related to *P. inflatum* and there is therefore some doubt as to the correct taxonomic placement of *Penicillium inflatum* (see Figure 3). Since several species in *Aspergillus* section *Versicolores* and the closely related section *Nidulantes* produce sterigmatocystin we examined the ex type culture of *A. tardus* for this mycotoxin. However we could not detect it under the conditions applied. No other species in section *Cremei*, including *Chaetosartorya* species and *Aspergillus wentii*, could produce sterigmatocystin.

Using HPLC-UV-FLD, LC-HRMS and LC-MS/MS we additionaly found aflatoxin $B_1$ from *A. togoensis* in a 14 days old extract of incubated on YES at 25°C. The isolate examined was CBS 205.75, the very same investigated by Wicklow et al. in 1989, but the previous study analyzed a sclerotial extract of this isolate cultivated on oat meal (Wicklow et al., 1989). Wicklow also examined a different isolate of *A. togoensis* (NRRL 13550) and fermented it on steam-sterilized rice for 6 weeks, and though no sclerotia were observed, large quantatives of sterigmatocystin was produced. The discovery of aflatoxin production in *A. stibothamnium* consolidate the species relationship with *A. flavus* (Samson and Seifert, 1985).

**Chemoconsistency**

We have not yet analyzed a strain of *A. versicolor* or *E. nidulans* that did not produce sterigmatocystin, but the frequency of the other species is more difficult to assess in a meaningful way, as there are often only few strains known for each of the other species.

The numerous publications for this research field have led to quite a few incorrect publications about sterigmatocystin- (Dean, 1963; Wilson et al., 2002; Maskey et al., 2003) and aflatoxin- (Hodges et al., 1964; Kulik and Holaday, 1966) production in *Penicillium* sp. The report of sterigmatocystin production by *P. luteum* is difficult to validate, as the original isolates is not available, and it is questionable which species Dean (Dean, 1963) was working with. We have checked *Talaromyces luteus* for sterigmatocystin and could not find it in any isolate of that species. Results reported by Wilson et al. (2002) that *P. griseofulvum*, *P. camemberti* and *P. commune* should be able to produce sterigmatocystin were an unfortunate swap of cyclopiazonic acid with sterigmatocystin (Frisvad et al., 2006). In the case of *P. chrysogenum*, the isolate tested was claimed to produce sterigmatocystin and 7 other derivates or precursors of sterigmatocystin (Maskey et al., 2003). However, we did examine this culture and it was a mixed culture of *P. chrysogenum* and *A. versicolor*, and it was the *A.*

*versicolor* that actually could produce sterigmatocystin and derivatives. These findings still have a tendency to re-occur in review literature and databases, and we hereby wish to clarify this; see Varga et al. (2009) and Frisvad et al. (2006) for reviews on incorrect assignments.
It is important to notice, however, that some of these species may be later shown to produce sterigmatocystin using other conditions than we used or using other strains than we used. For example *Eurotium amstelodami* and *Bipolaris sorokiniana* have been reported to produce sterigmatocystin several times latest in Barnes et al. (Barnes et al., 1994). We did examine several cultures of those two species and found no sterigmatocystin being produced them, but we have not examined *E. amstelodami* SRRC 10 and *Bipolaris sorokiniana* MRC 93 yet. Likewise the original producer of sterigmatocystin of *Monocillium nordinii* was not available in any culture collection, so we only able to examine some other strains of this species. In those we found no sterigmatocystin production on CYA, YES, OAT or WATM agars. The strains of *Aschersonia coffeae* and *Moellerialle reineckiana* reported to produce sterigmatocystin (Isaka et al., 2009) are not available yet from any culture collection, so these could not be checked in this study.

**Phylogenetics**
The evolutionary history was inferred using the Maximum Parsimony method (Eck and Dayhoff, 1966). Tree #1 out of 69 most parsimonious trees (length = 473) is shown. The consistency index is (0.635870), the retention index is (0.860125), and the composite index is 0.615937 (0.546927) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein, 1985). The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 (Nei and Kumar, 2000; Felsenstein, 1985) in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale (see Figure 4), with branch lengths calculated using the average pathway method (see pg. 132 in Nei and Kumar (2000)) and are in
the units of the number of changes over the whole sequence. All alignment gaps were treated as missing data. There were a total of 503 positions in the final dataset, out of which 139 were parsimony informative. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007; Hillis and Bull, 1993; White et al., 1990).

The phylogenetic analysis clearly illustrates the vast diversity spanned by the sterigmatocystin producing species and it is possible that the sterigmatocystin biosynthesis have independently evoloved 2-3 times within *Aspergillus* (section *Flavi*, *Nidulantes* and *Ochraceorosei*) and 4 times in the unrelated genera of *Bipolaris*, *Chaetomium*, *Humicola* and *Monocillium*.

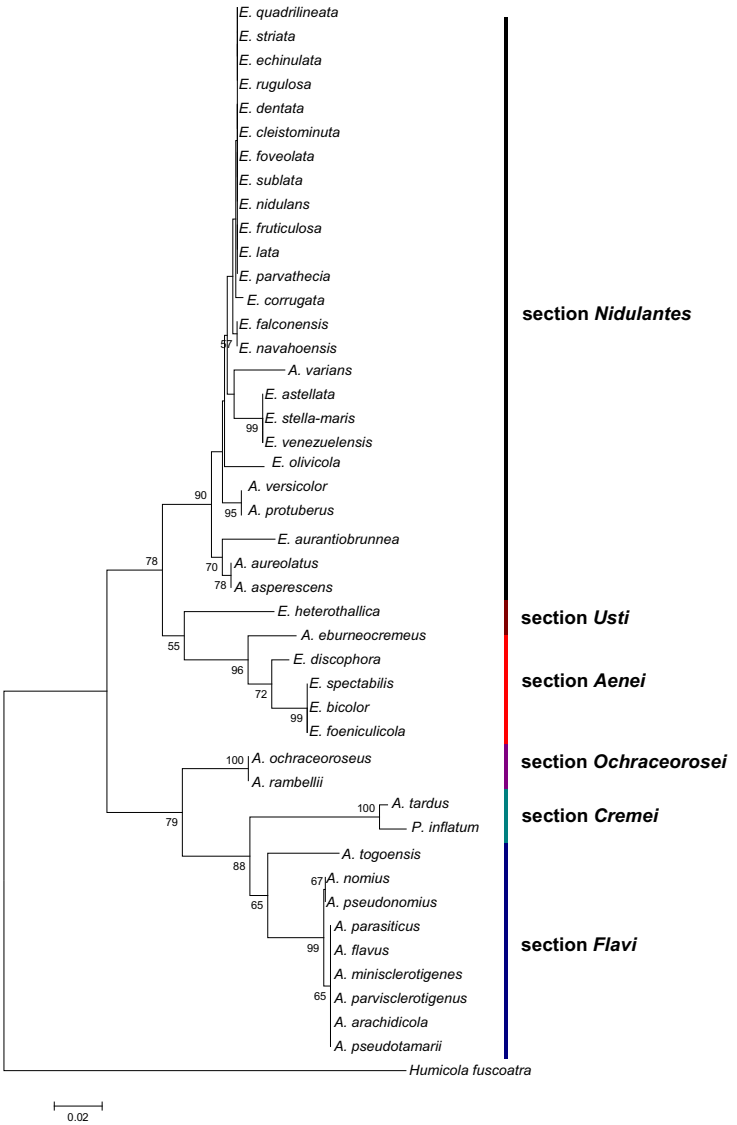


**Figure 4.** Maximum parsimony tree based on ITS sequence data of Aspergillus species able to produce sterigmatocystin. Numbers above branches are bootstrap values. Only values above 70% are indicated. Section *Usti* and *Aenei* are likely to be merged with section *Nidulantes*, thus the corresponding species are added to section *Nidulantes* in Table 1.

**Table 1.** Species verified as sterigmatocystin (and sometimes aflatoxin) producers.

| Species | Strain [a] | Aflatoxin | O-M-sterigmatocystin | Sterigmatocystin | Reference |
|---|---|---|---|---|---|
| ***Aspergillus* subgenus *Nidulantes*** | | | | | |
| **Section *Nidulantes* (Teleomorph *Emericella*)** | | | | | |
| *Em. acristata* | IMI 061453 | *0* | *0* | *X* | (Frisvad, 1985; Horie and Yamazaki, 1985; Horie et al., 1989) |
| *Em. astellata* | CBS 261.93<br>**WB 2396** | *X* | *0* | *X* | (Frisvad et al., 2004; Frisvad et al., 2005) |
| *Em. aurantiobrunnea* | CBS 465.65 | *0* | *0* | *X* | (Rabie et al., 1977) |
| *A. aureolatus* | WB 5126 | *0* | *0* | *X* [c] | Reported here |
| *Em. bicolor* | CBS 425.77 | *0* | *0* | *X* | (Frisvad, 1985) |
| *Em. cleistominuta* | CBS 200.75 | *0* | *0* | *X* | (Frisvad, 1985) |
| *Em. corrugata* | CBS 191.77 | *0* | *0* | *X* | (Horie and Yamazaki, 1985; Frisvad, 1985; Fujimoto et al., 1998) |
| *Em. dentata* | CBS 114.63 | *0* | *0* | *X* | (Horie and Yamazaki, 1985; Frisvad, 1985; Horie et al., 1989; Yamazaki et al., 1980; Horie et al., 1979) |
| *Em. discophora* | **CBS 469.88**<br>CBS 470.88 | *0* | *0* | *X* | (Zalar et al., 2008) |
| *A. eburneocremeus* | IMI 069856 | *0* | *0* | *X* [c] | Reported here |
| *Em. echinulata* | CBS 120.55<br>**CBS 292.80**<br>CBS 654.80 | *0* | *0* | *X* | (Frisvad, 1985; Horie et al., 1989) |
| *A. egyptiacus* | CBS 991.72 | *0* | *0* | *X* | (Moubasher et al., 1977) |
| *Em. falconensis* | NHL 2999 | *0* | *0* | *X* | Reported here |
| *Em. foeniculicola* | CBS 156.80 | *0* | *0* | *X* | (Horie and Yamazaki, 1985; Frisvad, 1985) |
| *Em. foveolata* | CBS 279.81 | *0* | *0* | *X* | (Frisvad, 1985) |
| *Em. fructiculosa* | CBS 989.72<br>CBS 650.73C<br>**CBS 650.77C** | *0* | *0* | *X* | (Frisvad, 1985) |
| *Em. heterothallica* | **WB 5097**<br>WB 5096<br>**WB 4981**<br>WB 4983 | *0* | *0* | *X* | (Horie and Yamazaki, 1985; Frisvad, 1985) |
| *Em. lata* | **CBS 492.66**<br>IMI 126693 | *0* | *0* | *X* | (El-Khady and Hafez, 1981; Frisvad, 1985; Horie et al., 1989; Yamazaki et al., 1980; Horie et al., 1979; Horie and Yamazaki, 1985) |
| *Em. navahoensis* | IMI 259711 | *0* | *0* | *X* | (Frisvad, 1985) |
| *Em. nidulans* | Confirmed in all strains examined (>100) | *0* | *0* | *X* | (Schroeder and Kelton, 1975; Horie and Yamazaki, 1985; Holzapfel et al., 1966; Engel and Teuber, 1980; El-Khady and Hafez, 1981; Chung et al., 1989; Hajjar et al., 1989; Moubasher et al., 1977; Barnes et al., 1994; Frisvad, 1985; Gedek, 1977; Soboleva and Kurmanov, 1984; |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | Horie et al., 1989; Horie et al., 1979; Ishida et al., 1972; Rabie et al., 1977) |
| *Em. olivicola* | **IMI 053749**<br>CBS 597.65<br>IMI 136777<br>CBS 119.37 | *0* | *0* | *X* | (Zalar et al., 2008) |
| *Em. parvathecia* | CBS 493.65 | *0* | *0* | *X* | (Horie and Yamazaki, 1985) |
| *Em. quadrilineata* | **CBS 591.65A**<br>CBS 125.55<br>CBS 128.49<br>CBS 235.65<br>IMI 089349<br>RMF DMG 936<br>IMI 139349 | *0* | *0* | *X* | (Horie and Yamazaki, 1985; Rabie et al., 1977; El-Khady and Hafez, 1981; Gbodi, 1993; Barnes et al., 1994; Frisvad, 1985; Horie et al., 1989) |
| *Em. rugulosa* | **CBS 133.60**<br>**CBS 117.50** | *0* | *0* | *X* | (Schroeder and Kelton, 1975; Horie and Yamazaki, 1985; Rabie et al., 1977; El-Khady and Hafez, 1981; Ballantine et al., 1965; Frisvad, 1985; Horie et al., 1989) |
| *Em. spectabilis* | CBS 429.77A | *0* | *0* | *X* | (Horie and Yamazaki, 1985; Frisvad, 1985) |
| *Em. stella-maris* | CBS 113638 | *0* | *0* | *X* | (Zalar et al., 2008) |
| *Em. striata* | **CBS 451.75**<br>CBS 866.70 | *0* | *0* | *X* | (Horie and Yamazaki, 1985) |
| *Em. venezuelensis* | CBS 868.97 | *X* | *0* | *X* | (Frisvad and Samson, 2004; Frisvad et al., 2005) |
| **Section *Versicolores*** | | | | | |
| *A. asperescens* | IMI 046813 | *0* | *0* | *X* [c] | Reported here |
| *A. protuberus* | CBS 658.74 | *0* | *0* | *X* [c] | Reported here |
| *A tardus* | **CBS 576.95B**<br>CBS 576.95A | *0* | *0* | *0* | Reported here |
| *A. versicolor* | Confirmed in all strains examined (>200) | *0* | *0* | *X* [c] | (Birkinshaw and Hammady, 1957; Holker and Mulheirn, 1968; Davies et al., 1956; Davies et al., 1960; Hatsuda et al., 1954; Hatsuda and Kuyama, 1954; Holker and Kagal, 1968; Engel and Teuber, 1980; Moubasher et al., 1977; Barnes et al., 1994; Gedek, 1977; Chelkowski et al., 1979; Soboleva and Kurmanov, 1984) |
| ***Aspergillus* subgenus *Circumdati*** | | | | | |
| **Section *Flavi* (*Petromyces*)** | | | | | |
| *A. arachidicola* | **CBS117610** | *X(B+G)* | *X* | *(X)* [c] | (Pildain et al., 2008) |
| *A. bombycis* | NRRL 26010[T]<br>**NRRL 25593**<br>NRRL 29253 | *X(B+G)* | *X* | *(X)* [c] | (Peterson et al., 2001; Frisvad et al., 2005) |
| *A. flavus* | NRRL 3357<br>NRRL 1957 | *X*<br>*0* | *X*<br>*0* | *(X)/X*<br>*0* | (Kulik and Holaday, 1966; Schroeder and Kelton, 1975; Barnes et al., 1994; Moubasher et al., 1977) |
| *A. nomius* | **CBS 260.88**[T]<br>NRRL 25393 | *X(B+G)* | *X* | *(X)* [c] | (Kurtzman et al., 1987; Frisvad et al., 2005) |

| | | | | | |
|---|---|---|---|---|---|
| *A. nomius* (*A. zhaoquingensis*) | CBS 399.93 | *X($B_2$)* | *X* | *(X)* [c] | (Frisvad et al., 2005) |
| *A. parasiticus* | CBS 100926 | *X(B+G)* | *X* | *(X)* [c] | (Kulik and Holaday, 1966; Schroeder and Kelton, 1975; Hsieh et al., 1973; Moubasher et al., 1977; Barnes et al., 1994; Atalla et al., 2003) |
| *A. parvisclerotigenus* | CBS 121.62 | *X(B+G)* | *X* | *(X)* [c] | (Saito and Tsuruta, 1993; Frisvad et al., 2005) |
| *A. pseudotamarii* | **CBS 766.97$^T$**<br>CBS 765.97 | *X* | *X* | *(X)* [c] | (Ito et al., 2001; Frisvad et al., 2005) |
| *A. togoensis* | CBS 205.75 | *X* | *X* | *X* | (Wicklow et al., 1989); aflatoxin $B_1$ production reported for the first time here |
| *A. toxicarius* | **CBS 822.72$^T$**<br>CBS 561.82 | *X* | *X* | *(X)* [c] | (Rigó et al., 2002; Frisvad et al., 2005) |
| **Section *Ochraceorosei*** | | | | | |
| *A. ochraceoroseus* | CBS 550.77 | *X* | *X* | *X* | (Klich et al., 2000; Frisvad et al., 1999; Frisvad et al., 2005) |
| *A. rambellii* | ATCC 42001 | *X* | *X* | *X* | (Frisvad et al., 2005) |
| ***Chaetomium*** | | | | | |
| *Chaetomium* sp. | - | | | | (Rabie et al., 1976; Maes and Steyn, 1984; Udagawa et al., 1979b) |
| *C. cellulolyticum* | CBS 547.75 | *0* | *X* | *X* | (Barnes et al., 1994; Sekita et al., 1981b) |
| *C. longicolleum* | CBS 103.79<br>CBS 119.57A<br>**CBS 119.57B** | *0* | *X* | *X* | (Koyama et al., 1991) |
| *C. malaysiense* | CBS 760.83 | *0* | *X* | *X* | Reported here |
| *C. virescens*<br>*= Achaetomiella virescens* | NRRL 25287 | *0* | *X* | *X* | (Sekita et al., 1981a) |
| *C. udagawae* | CBS 337.68 | *0* | *0* | *X* | (Udagawa et al., 1979a) |
| ***Penicillium*** | | | | | |
| *P. inflatum* | FRR 3612<br>CBS 576.95B | *0* | *0* | *X* | Reported here |
| ***Humicola*** | | | | | |
| *H. fuscoatra* | NRRL 22980 | *0* | *0* | *X* | (Joshi et al., 2002) |

[a] Strongest producing isolate in our studies marked in bold.
[b] Reported here for the first time.
[c] By implication, as aflatoxin is reported to be produced.
Measurements of aflatoxin and sterigmatocystin based on HPLC-UV, LC-HRMS and MSMS detection.

Table 2. Species in which sterigmatocystin or aflatoxin production could not be confirmed

| Species | Strain [b] | Aflatoxin | Sterigmatocystin | Reference |
|---|---|---|---|---|
| *Sterigmatocystis* sp.[b]<br>(= *Aspergillus* sp.) | - | *0* | *X* | (Nekam and Polgar, 1948) |
| *A. carneus* | - | *0* | *0* | (Moubasher et al., 1977) |

| | | | | |
|---|---|---|---|---|
| *A. flavipes* | NRRL 32623 | *0* | *0* | (Moubasher et al., 1977) |
| *A. fumigatus* | CBS 144.89 | *0* | X | (Hasan, 1993) |
| *A. multicolor* | - | *0* | *X* | (Rabie et al., 1977; Hamasaki et al., 1980; Frisvad, 1985) |
| *A. implicatus* | CBS 484.95 | *0* | *0* | Tested for sterigmatocystin production because other Aspergilli find in the Taï National Forest, Ivory Coast produced sterigmatocystin |
| *A. sydowii* | IBT 29116 | *0* | *X* | (Wyllie and Morehouse, 1975); Davis, 1981; Lisker, 1993???? |
| *A. tamarii* | | | | (Moubasher et al., 1977; Goto et al., 1996; Ito et al., 1998) |
| *A. ustus* | CBS 114901 | *0* | *0* | (Moubasher et al., 1977; Rabie et al., 1977) |
| *Em. variecolor* | CBS 261.93 | *0* | X | (Chexal et al., 1975; Moubasher et al., 1977) |
| *A. wentii* | CBS 229.67 | *X* | *0* | (Kulik and Holaday, 1966) |
| *Bipolaris sorokiniana* | KVL 1623 | *0* | *X* | (Holzapfel et al., 1966; Barnes et al., 1994; Maes and Steyn, 1984) |
| *Farrowia* sp. (= *Chaetomium* sp.) | | *0* | *X* | (Udagawa et al., 1979b) |
| *C. gracile* | CBS 146.60 | *0* | *X* | (Sekita et al., 1981b) |
| *C. caprinum* | | *0* | *X* | (Sekita et al., 1981a) |
| *C. tetrasporum* | CBS 351.77 | *0* | *X* | (Sekita et al., 1981b) |
| *C. thielavioideum* | | *0* | *X* | (Udagawa et al., 1979b; Barnes et al., 1994; Sekita et al., 1981b; Sekita et al., 1980) |
| *Em. purpurea* | CBS 754.74 | *0* | *X* | (Horie and Yamazaki, 1985) |
| *Em. unguis* | SRRC 271 | *0* | *X* | (Barnes et al., 1994; Mislivec et al., 1975) |
| *Em. violacea* | CBS 138.55 | *0* | *X* | (El-Khady and Hafez, 1981) |
| *Fusarium kyushuense* | NRRL 3509 | *X* | (X)[c] | (Schmidt-Heydt et al., 2009) |
| *Monocillium nordinii* | CBS 147.70 | *0* | X | (Ayer et al., 1981) |
| ***Aspergillus* section *Aspergillus* (Teleomorph *Eurotium*)** | | | | |
| *Eu. amstelodami* | | *0* | *0* | (Schroeder and Kelton, 1975; El-Kady et al., 1994; Ahmed et al., 2005; Barnes et al., 1994) |
| *Eu. chevalieri* | | *0* | *X/0* | (El-Kady et al., 1994; Ahmed et al., 2005; Barnes et al., 1994; Karo and Hadlok, 1982; Schroeder and Kelton, 1975; Ahmed et al., 2005; Moubasher et al., 1977) |
| *Eu. intermedium* | | *0* | *X* | (El-Kady et al., 1994) |
| *Eu. pseudoglaucum* | | *0* | *X* | (El-Kady et al., 1994; Soboleva and Kurmanov, 1984) |
| *Eu. repens* | | *0* | *0* | (Szebiotko et al., 1981; Soboleva and Kurmanov, 1984; Karo and Hadlok, 1982) |

| | | | |
|---|---|---|---|
| *Eu. rubrum* | *0* | *X/0* | (El-Kady et al., 1994; Barnes et al., 1994; Karo and Hadlok, 1982) (Kulik and Holaday, 1966; Schroeder and Kelton, 1975; Leitao et al., 1989) |

**Experimental part**

**Chemicals.** All solvents used were HPLC grade from Sigma-Aldrich (St. Louis, MO, USA). Standards of aflatoxins $B_1$ $B_2$, $G_1$ and $G_2$ as well as and sterigmatocystin and 3-O-methylsterigmatocystin were also from Sigma-Aldrich. Water used was Milli-Q grade (Millipore, Bedford, MA).

**Fungal strains and extraction.** Cultures were inoculated for 1 week and 2 weeks at 25°C in the dark on Czapek yeast autolysate (CYA) agar (Samson et al., 2004), WATM agar (Wickerhams Antibiotic Test Medium) (Raper and Thom, 1949), Yeast Extract Sucrose (YES) agar with Biokar (Biokar Diagnostics, Beauvais, France) yeast extract, Malt Extract Autolysate (MEA) agar according to Blakeslee (Raper and Thom, 1949) (with Difco malt extract) and oat meal (OAT) agar, made with 30 g Danish organic oat meal. An additional set of cultures on CYA were additionally incubated for 1 and 2 weeks at 37°C. CBS cultures (Table 1 and 2) are available from CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; and IBT cultures from the IBT Culture Collection at DTU Systems Biology, Technical University of Denmark. Extracts made by the micro extraction procedure of Smedsgaard (Smedsgaard, 1997) where ~0.6 $cm^2$ plugs of culture were extracted with 1000 µl methanol-dichlormethane-ethyl acetate (1:2:3 v/v) and 1% formic acid, which was decanted into a new vial and evaporated in vacuo. The dry extracts were redissolved 400 µl methanol filtered through a PFTE filter.

**Analysis of extracts.** Microextracts, 1-3 µL, were analyzed by 3 different methods: **1)** HPLC with UV/Vis-diode array detection (200-600 nm) and fluorescence detection (FLD) set at 365→428 and 365→450 nm. Separation was done on a 10 cm, 3µm, Luna C18(2) column (Phenomenex, Torrence, CA) using a water-acetonitrile ($CH_3CN$) gradient from 15% $CH_3CN$ to 100% $CH_3CN$ in 20 min with bots solvents buffered with 50 ppm trifluoroacetic acid (Jennessen et al., 2005). **2)** Exact mass HPLC-DAD-HRMS was done on a similar LC system but with a 5 cm column and 20 mM formic acid as buffer. MS was a LCT oaTOF mass spectrometer (Micromass, Manchester, UK) as described in Nielsen and Smedsgaard (2003) and Nielsen
et al. (2009). **3)** HPLC-MS/MS was done as on a similar system as above but with the LC coupled to a Micromass Ultima triple-quadropole mass spectrometer with the MS settings described in Nielsen et al. (2009). Extracts were separated on a Phenomenex Gemini $C_6$-phenyl, 3µm, 2 × 50mm column with a flow of 300 µL/min from 20 to 90% $CH_3CN$ over 15min, increased to 500 µL/min from 90 to 100% $CH_3CN$ in additional 1 min. The column was washed from 2 min with 100% $CH_3CN$ at 500 µL/min and

returned to the start conditions in 5 min. MS/MS settings can be seen in table 3. LC-MS/MS data was evaluated using the Micromass Quanlynx 4.1 software.

Table 3. MS/MS method including retention times, transition ions and the cone and collision energies used.

| Compound | RT (min) | Ion type | Transition (*m/z*)[a] | Cone (V) | Collision energy (eV) |
|---|---|---|---|---|---|
| Aflatoxin $B_1$ | 6.0 | Quantifier | 313 → 285 | 25 | 40 |
| | | Qualifier | 313 → 241 | 25 | 40 |
| 3-O-Methyl-sterigmatocystin | 8.3 | Quantifier | 339 → 306 | 25 | 50 |
| | | Qualifier | 339 → 295 | 25 | 50 |
| Sterigmatocystin | 9.8 | Quantifier | 325 → 310 | 25 | 40 |
| | | Qualifier | 325 → 281 | 25 | 40 |

[a]All transitions were made from $[M+H]^+$.

**NMR validation of sterigmatocystin (ST) production in the *Penicillium inflatum.*** The isolate (IBT 22948 = FRR 3612, from soil, Mt. Albert, Victora, Australia) used for isolation of sterigmatocystin was obtained from the IBT Culture Collection at DTU Systems Biology, Technical University of Denmark. The isolate was cultured grown for 14 days at 25°C on 20 Petri dishes with yeast extract sucrose agar (YES). The strain is described by Stolk and Malla (1971). The plates were homogenized using a Stomacher and 20 mL ethyl acetate with 1% formic acid. The extract was filtered and dried down on a freeze drier. The crude extract was separated in three phases by swirling 15 mL of 1: heptane, 2: dichloromethane (DCM) and 3: MeOH. Analytical LC-DAD-FLD showed that ST was most concentrated in the DCM phase (17 mg). ST was further purified by preparative Waters HPLC W600/996PDA (Milford, MA, USA) on a Waters Delta Pack 300 x 19 mm, 15 µm, C-18 column using a gradient of 55% $CH_3CN$-$H_2O$) to 65% over 10 min buffered with 50ppm trifluoroacetic acid. The collected fractions were combined and concentrated in-vacuo and dried down under $N_2$ to yield 8 mg of yellow, crystalline ST. UV absoption was measured with a Shimadzu Spectrophotometer UV-VIS 240 (Shimadzu, Japan). UV/Vis ($CH_3CN$): $\lambda_{max}$ (log ε) 206 (3.86); 250 (3.88); 328 (3.84). NMR spectra were acquired in $CDCl_3$ on a Varian Inova Unity spectrometer. $^1$H NMR (499.87 MHz, $CDCl_3$, 25 °C, 7.24 ppm): 13.2 (s, 1H, OH), 7.48 (t, $^3J$ (H,H)=8.3, 1H, CH), 6.82 (d, $^3J$ (H,H)=7.3, 1H,
CH), 6.81 (s, 1H, CH), 6.74 (d, $^3J$ (H,H)=7.3, 1H, CH), 6.49 (s, 1H, CH), 6.43 (s, 1H, CH), 5.43 (s, 1H, CH), 4.80 (d, $^3J$ (H,H)=7.3, 1H, CH), 3.98 (s, 3H, $OCH_3$); $^{13}$C NMR (125.71, $CDCl_3$,77.23 ppm, 25 °C, 39.4 ppm): 180.9, 164.1, 162.8, 161.8, 154.5, 153.7, 145.4, 135.8, 113.3, 111.3, 108.5, 106.0, 105.9, 105.4, 102.5, 90.5, 56.8, 48.1.

**Genotypic analysis.** The cultures used for the molecular studies were grown on malt peptone (MP) broth using 10 % (v/v) of malt extract (Oxoid) and 0.1 % (w/v) bacto peptone (Difco), 2 mL of medium

in 15 mL tubes. The cultures were incubated at 25 °C for 7 days. DNA was extracted from the cells using the Masterpure™ yeast DNA purification kit (Epicentre Biotechnol.) according to the instructions of the manufacturer. Fragments containing the ITS region were amplified using primers ITS1 and ITS4 as described previously (White et al., 1990). Sequence analysis was performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit for both strands, and the sequences were aligned with the MT Navigator software (Applied Biosystems). All the sequencing reactions were purified by gel filtration through Sephadex G-50 (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in double-distilled water and analyzed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

DNA sequences were edited with the DNASTAR computer package. Alignment and phylogenetic analysis of sequence data were performed using MEGA version 4 (Tamura et al., 2007). Alignment gaps were treated as fifth character state, parsimony uninformative characters were excluded and all characters were unordered and equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option. To assess the robustness of the topology, 1000 bootstrap replicates were run by maximum parsimony (Hillis and Bull, 1993). Other measures including tree length, consistency index, retention index and rescaled consistency index (CI, RI and RC, respectively) were also calculated.

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## Paper 6

### Comparative chemistry of *Aspergillus oryzae* (RIB40) and *A. flavus*

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# Comparative chemistry of *Aspergillus oryzae* (RIB40) and *A. flavus*

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**Abstract**

The great importance of *Aspergillus oryzae* and *A. flavus* in industrial biotechnology and food safety has been the reason why strains of these two species were some of the first aspergilli to be fully genome sequenced. Bioinformatic analysis has revealed a homology of ca. 99,5% at the genomic level and ca. 98,0% at the protein level between the two species together pointing towards a large homology also in secondary metabolite production. The present study reports the first comparison of secondary metabolite production between the full genome sequenced strains of *A. oryzae* (RIB40) and *A. flavus* (NRRL 3357). Surprisingly the overall chemical profiles of the two strains were often very different, when extracts from 15 different medium compositions were compared. The fact that we found the aflatrem precursor 13-desoxypaxiline to be a major metabolite and biosynthetic pathway end product in *A. oryzae* indicates that regulation of the biosynthetic pathway is very different in the two species or that a *A. oryzae* carries a mutation in the genes responsible for the final step. The present paper also for the first time reports parasiticolide A and two new analogues from *A. oryzae.*

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**Introduction**

*Aspergillus oryzae* is one of industry's most used "workhorses" and has been used for centuries in food fermentation for the production of e.g. sake, soy sauce and other traditional Asian foods (Machida et al., 2008b). *A. oryzae* is also a widely used organism for production of amylase, lipases and proteases and more recently non-fungal proteins (Punt et al., 2002; Meyer, 2008). For many years, *A. oryzae* has been suspected to be a domesticated form of *A. flavus*, a plant and mammalian pathogenic saprophyte, capable of producing some of the most carcinogenic compounds known: the aflatoxins. Genetic work and subsequent genome sequencing of strains of both species have verified

the tight link between the species (Geiser et al., 1998; 2000; Abe et al., 2006; Kobayashi et al., 2007; Machida et al., 2008b).

The relationship of the two species has resulted in extensive screening of the toxic potential of *A. oryzae*, but no genuine evidence of aflatoxin production in validated *A. oryzae* isolates has ever been shown. Other important toxins, known from *A. flavus*, have on the other hand been shown in *A. oryzae*: 3-nitropropionic acid (Iwasaki and Kosikowski, 1973; Orth, 1977) and cyclopiazonic acid (CPA) (Orth, 1977) along with kojic acid (Manabe et al., 1984; Bentley, 2006). Additional metabolites previously reported from *A. oryzae* are asperfuran (Pfefferle et al., 1990), sporogen AO1 (Tanaka et al., 1984a; 1984b), maltoryzine (Iizuka and Iida, 1962), and aspergillomarasmine (Lederer, 1963; Robert et al., 1962). The aspirochlorines (= A30416 = oryzaechlorin (Monti et al., 1999)) have been found in both *A. flavus*, *A. oryzae* and *A. tamarii* (Sakata et al., 1982; 1983; 1987). For reviews on the safety of *A. oryzae*, see Barbesgaard et al. (1992), Tanaka et al. (2002) and Abe et al. (2006).

The few predicted differences between the genomes of *A. oryzae* and *A. flavus* (ca. 99.5% genome homology and 98% at the protein level for RIB40/ATCC 42149 and NRRL 3357 (Rokas et al., 2007)), could lead one to expect *A. oryzae* to produce most of the metabolites found in *A. flavus* (Machida et al., 2005; 2008a; 2008b; Payne et al., 2006; Yu et al., 2008), but published metabolic data indicates a very low chemical correlation (Laatsch, 2009). It is with reference to the established genetic heritage of *A. oryzae* from *A. flavus* remarkable that maltoryzine, sporogen AO1, asperfuran and aspergillomarasmine never have been truly identified in *A. flavus*. Though research on *A. flavus* chemistry has been focused primarily on toxic compounds, these metabolites should be part of its chemical potential as they are for the domesticated *A. oryzae*. The preliminary bioinformatic studies in conjunction with the genome sequencing shows roughly the same number of predicted genes: 32 polyketide synthases (PKSs) and 28 non-ribosomal synthases (NRPSs) for *A. flavus* and 32 PKSs and 27 NRPSs for *A. oryzae* with 2 NRPSs apparently unique for each strain (Cleveland et al., 2009). The exclusiveness of these genes in terms of end product has yet to be verified chemically.

Most of the predicted genes for secondary metabolites of *A. oryzae* (or *A. flavus*) have not been mapped to specific metabolic products, despite the genome sequencing of RIB40 in 2005 (Machida et al., 2005).

Only genes of the most important toxins: aflatoxin (Lee et al., 2006; Tominaga et al., 2006; Yu et al., 2008), CPA (Tokuoka et al., 2008; Chang et al., 2009) and aflatrem (Nicholson et al., 2009) have been fully annotated in both species, which leaves much to be explored. The full chemical potential of either species is unknown and epigenetic modifiers (Shwab et al., 2007; Shwab and Keller, 2008; Williams et al., 2008; Henrikson et al., 2009) may be necessary, alongside with the use of different growth conditions to aid triggering the full potential of secondary metabolite expression in these two closely related species.

The aim of the current work has been to perform a comparative investigation of the chemistry from the two genome sequenced strains of respectively *Aspergillus oryzae* (RIB40) and *A. flavus* (NRRL

3357), in order to get further insights into possible homologies in secondary metabolite production for these two important species.

**Results and Discussion**

**De-replication of *A. oryzae* RIB40**

For the analysis of *A. oryzae* RIB40 chemistry, we investigated a series of solid media (YES, YESBEE, DRYES, CYA, CYAS, CY20, CY40, DUL, GAK, GMMS, MEA, OAT, PDA, TGY, WATM (see Methods and Materials for explanation) cultivations with micro-scale extractions (Frisvad and Thrane, 1987; Smedsgaard, 1997) and subsequently analyzed with HPLC-DAD-MS for selection of optimal conditions. The different media were tested on a collection of *A. oryzae* (RIB40, IBT 28103) and *A. flavus* (NRRL 3357, IBT 23106, IBT 3642) and these strains were cultivated at 25°C in dark for 7 and 14 days. The media screening for *A. oryzae* and *A. flavus* indicated the greatest chemodiversity and metabolite production from the CYA, YES and WATM agar for our purpose.

The comparison of the chemical profiles of the two strains, NRRL 3357 and RIB40, exposed a surprisingly high degree of chemical difference on all media as illustrated in Figure 1 and Table 1 for the WATM medium. The major metabolite repetitions between the two genome sequenced strains were merely kojic acid and ergosterol. This is in sharp contrast to the high gene homology, particularly for the secondary metabolite genes.

Table 1. LC-HRMS de-replication of some of the important metabolites from the two full genome sequenced siblings, *A. flavus* (NRRL 3357) and *A. oryzae* (RIB40). Based on 7 day fermentation on solid WATM. Parenthesis indicates trace amounts.

| Metabolite | *A. flavus* NRRL 3357 | *A. oryzae* RIB40 |
|---|---|---|
| Kojic acid | + | + |
| Aflatoxin $B_1$ | + | - |
| Aflatoxin $B_2$ | + | - |
| Aflavarin | (+) | - |
| Aflavinines | (+) | + |
| Aflatrem | + | - |
| 13-desoxypaxiline | (+) | + |
| Aspriochlorine | - | + |
| Cyclopiazonic acid | + | - |
| Ditryptophenaline | + | - |
| 14-deacetyl parasiticolide A | - | + |

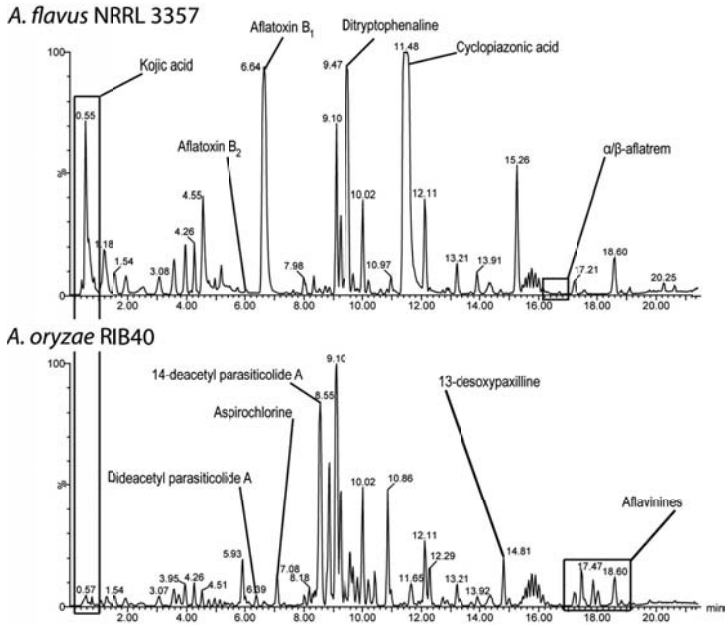



**Figure 1**. ESI$^+$ BPI chromatogram of 7 day micro scale extract from WATM, bottom: *A. oryzae* RIB40, top: *A. flavus* NRRL 3357. Besides kojic acid and analogues in the beginning of chromatogram and ergosterol in the end (not shown), there is little chemical overlap between the genetically almost identical strains.

Known metabolites to *A. oryzae* was de-replicated and we found that the RIB40 strain did not produce detectable levels (LC-MS) of CPA (as also noted by (Tokuoka et al., 2008)), asperfuran, sporogen AO1, maltoryzine or aspergillomarasmine under these growth conditions. It did, however, produce kojic acid and aspirochlorine and a series of potentially new metabolites of which some were isolated and reported here.

**New metabolites to *A. oryzae* RIB40**

During fermentation of the chemical potent RIB40 strain, we have been interested in the tremorgenic compounds, allegedly coupled to fungal sclerotia (Wicklow and Cole, 1982; Gloer et al., 1988; 1989; Staub et al., 1992; 1993; Tepaske et al., 1989a; 1989b; 1990; 1992) and whether these could be found in *A. oryzae* as they have been in *A. flavus*. The RIB40 strain produces large and abundant sclerotia, especially on WATM agar, a fact not widely announced in literature although sclerotia have been observed in *A. oryzae* sporadically (Raper and Fennell, 1965; Wicklow et al., 2007; Jin et al., 2009), why the potential for sclerotium-coupled chemistry is present. No sclerotia were observable after 14 days on YES agar, but although these metabolites are often characterized as sclerotial metabolites, there is not a strict correlation between the biosynthesis of these

metabolites and the formation of sclerotia, as also noted by Wilson (Wilson, 1966), and this extract was used for the described isolations.

Here, we report the discovery of the aflatrem precursor 13-desoxypaxilline (13-dehydroxypaxilline) in *A. oryzae* RIB40, originally isolated from *Penicillium paxilli* (Springer et al., 1975; Longland et al., 2000; Bilmen et al., 2002; Sabater-Vilar et al., 2003; Sheehan et al., 2009). Aflatrem is known from *A. flavus* and was discovered by Wilson and Wilson in 1964 (Wilson and Wilson, 1964) and structure elucidated by Gallagher et al. in 1978/1980 (Gallagher and Wilson, 1978; 1980). 13-desoxypaxiline was present in YES, CYA, OAT and WATM agar 7 day old micro-scale extracts (see Figure 2).

From the 14 day old YES 200 plate extract used for isolation, 13-desoxypaxilline was recovered as an intermediate metabolite. LC-MS, LC-MS/MS and NMR data analysis confirmed the structure. Naturally the prospect of finding aflatrem itself was investigated, though no apparent peak was visible in HPLC-DAD data files. The use of a LC-MS/MS method further confirmed 13-desoxypaxilline as an end-product of *A. oryzae* RIB40 for the above cultivation conditions, since none of the proposed intermediate steps towards aflatrem could be detected (LC-MS/MS) and only one sample (WATM, 7d) showed traces of paspaline, a precursor for 13-desoxypaxilline (Figure 3).

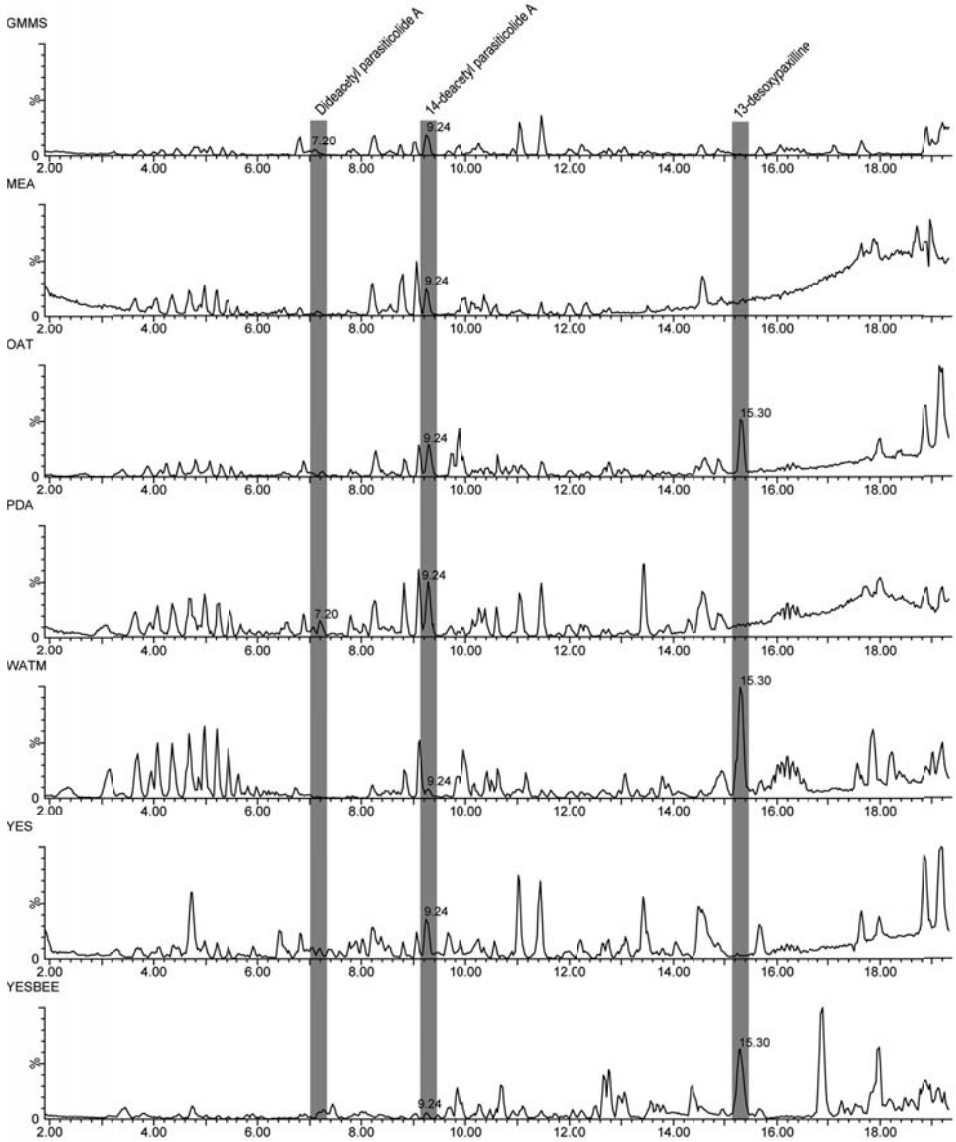



**Figure 2**. Selected media influence on the secondary metabolite production of *A. oryzae* RIB40 after 7 days cultivation on solid media at 25°C. Dide- and 14-deacetyl parasiticolide and 13-desoxypaxilline is seen at 7.20, 9.24 and 15.30 min (time shift compared to Figure 1) in the HPLC-MS (BPI+) chromatograms. Especially the production of 13-desoxypaxilline varies. The level of this metabolite is significantly higher after 14 days of incubation on YES agar.

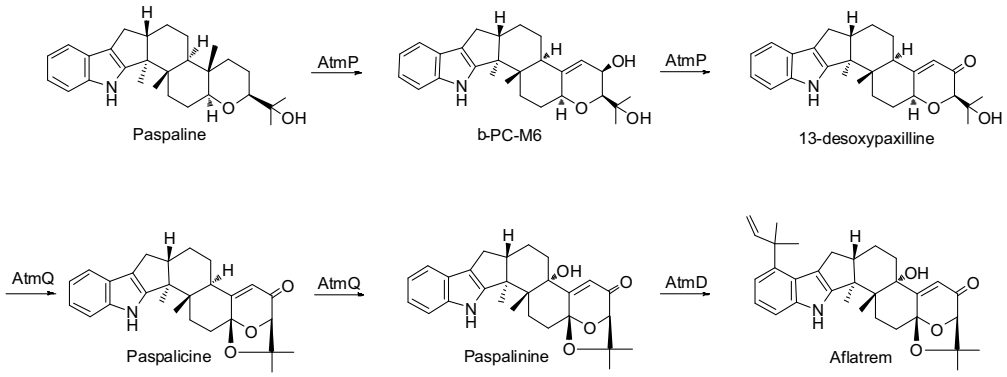



**Figure 3**. The final steps in the proposed biosynthesis of aflatrem (in *A. flavus*) though the stable *A. oryzae* intermediates: paspaline and 13-desoxypaxilline (Nicholson et al., 2009).

A second extract was made from 100 plates of a 14-day old *A. oryzae* RIB40 culture grown on WATM agar with abundant sclerotia formation to validate the findings from the YES extract. The analysis of the WATM extract showed 13-desoxypaxilline as a major metabolite alongside other sclerotia-related metabolites, such as aflavinines.

The discovery of 13-desoxypaxilline as the apparent end-product of *A. oryzae* RIB40 is in agreement with the recent analysis of Nicholson et al. (2009) who showed that a frameshift mutation in the *atmQ* gene presumably accounts for the conversion of 13-desoxypaxilline to paspalicine and paspalinine. This mutation is likely responsible for terminating the aflatrem biosynthesis in RIB40 prematurely, short of the acetal ring closure, C-13 hydroxylation and isoprene attachment. Contrary to our discovery, Nicholson et al. did not find the aflatrem gene cluster of RIB40 to be transcribed during their fermentations.

The isolated 13-desoxypaxilline is a member of the paspalitrem tremorgens, a widely distributed group of metabolites that have been isolated from several genera: *Penicillium*, *Eupenicillium*, *Claviceps, Emericella*, *Aspergillus* and *Phomopsis* (Cole et al., 1981; Steyn and Vleggaar, 1985; Bills et al., 1992). Besides the tremorgenic activity in animals, these metabolites have been shown to be insecticides (Laakso et al., 1993a; 1993b), which is believed to be their ecological function together with aflatoxin and CPA for protection of the sclerotia against fungivorous insects (Wicklow and Cole, 1982; Gloer et al., 1988).

A re-analysis of the fifteen different screenings media demonstrated much higher 13-desoxypaxilline production on YESBEE (and OAT) than for YES. This finding is in line with the hypothesis for ecological function described above, as YESBEE contains bee pollen and this could indicate an epigenetic key for

the aflatrem cluster of *A. flavus*/*A. oryzae*, since *A. flavus* occasionally and the more toxigenic *A. nomius* and *A. minisclerotigenes* (potential ancestors) predominantly are found on insects, soil and peanuts. This hypothesis remains to be proven in future studies.

In addition to 13-desoxypaxilline, two new analogues of parasiticolide A were also isolated and are here reported for the first time. The metabolites showed to be dide- and 14-deacetoxy analogues, and are most likely precursors to the sesquiterpene parasiticolide A (=astellolide A) (see Figure 4). The metabolites were present in CYA, YES and WATM extracts and isolated from the same 14 day old YES extract as 13-desoxypaxilline, and the dide- and 14-deacetyl analogues were also found in the sclerotia enriched 100 plate WATM extract. Again the metabolites were analyzed using LC-MS and NMR. Several different extraction procedures were tested to verify the correctness of the compounds as a genuine metabolite and not as *in vitro* degraded parasiticolide A products, but all samples showed only dide- and 14-deacetyl parasiticolide A and no traceable (LC-MS) levels of parasiticolide A itself, even with different non-acidic extractions. Parasiticolide A have been isolated from *A. flavus* var. *columnaris* (FKI-0739) once (Shiomi et al., 2002) and was originally isolated and characterized from *A. parasiticus* (IFO 4082) (Fukuyama et al., 1975; Hamasaki et al., 1975; Ishikawa et al., 1984) and later also a mycelia polyketide deficient mutant of *Emericella variecolor* (= *A. stellatus* Curzi) (Gould et al., 1981). Recently parasiticolides have been detected in the newly described species *A. arachidicola* (CBS 117610) and *A. minisclerotigenes* (CBS 117635) (Pildain et al., 2008). There have to our knowledge not been published any toxic studies on the parasiticolides, but the related peniopholides from the fungus *Peniophora polygonia* have been screened to be antifungal (Ayer and Trifonov, 1992).
In our observations parasiticolides are more often a detectable metabolites of *A. oryzae* than of *A. flavus* under the same fermentation conditions, suggesting that the pathway is partly silenced for *A. flavus* and may need epigenetic modification to be expressed under otherwise normal growth conditions. It is interesting that parasiticolide A is scarcely observed in *A. flavus*, when it is an important product of *A. oryzae* and also of *A. parasiticus*. As for 13-desoxypaxilline, dide and 14-deacetyl parasiticolide A are almost certainly products of a prematurely ended biosynthesis, here parasiticolide A. We also isolated and elucidated a third parasiticolide A analogue; a formyl variant of parasiticolide A, but it was not possible to exclude the possibility of *in vitro* chemistry due to the formic acid added during the ethyl acetate extraction, so the biological origin of this metabolite remains questionable. (Hamasaki et al. used benzene to extract parasiticolide A in 1975). To further verify these observations, an MS/MS method was used to analyze several different microscale extracts of RIB40 for parasiticolide A itself. Trace amounts of parasiticolide A was found under these conditions and compared to an isolate of *A. parasiticus* (IBT 4387) capable of producing parasiticolide A. In the *A. parasiticus* isolate no dide- or 14-deacetyl parasiticolide A could be measured, indicating a complete transformation into the end-product. The small amount of parasiticolide A in RIB40

(roughly 1:1000 ratio compared to 14-acetyl parasiticolide A, presuming the same response factor) might be the result of spontaneous acetylation involving the first acetylating enzyme. When the genecluster of this metabolite is mapped, it is likely that the gene responsible for the last (specific) acetylation will be found to be mutated.

Except for the section *Nidulantes* member *Emericella variecolor*, all other producers of these metabolites have been members of group *Flavi*. No indication points to these metabolites being part of the previous mentioned sclerotia metabolites since they are not found in selective sclerotia extracts, but is found for example in *A. arachidicola*, which is not known to produce sclerotia.

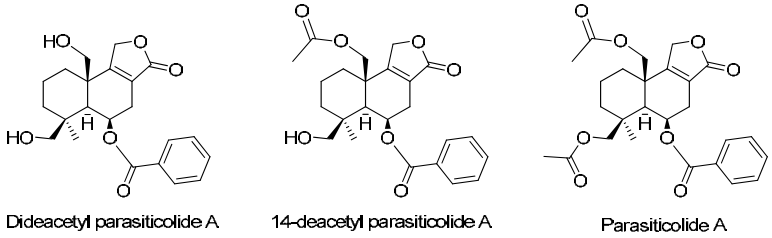



**Figure 4**. Dide- and 14-deacetyl parasiticolide A and parasiticolide A.

Along with the 13-desoxypaxilline and the two new parasiticolide analogues, we have observed several aflavinines in both YES and WATM extracts (Rank et al. unpublished). Aflavinines have previously been isolated from *A. flavus* (NRRL 6541) (Gloer et al., 1988) and several other Aspergilli (Gloer et al., 1989; Tepaske et al., 1989a; Laakso et al., 1993a).

**Conclusion**

The tremorgenic 13-desoxypaxilline has been isolated from *A. oryzae* RIB40 and verified under several growth conditions. We believe that 13-desoxypaxilline is the end-product of the aflatrem biosynthesis for the RIB40 strain since no aflatrem could be detected in any of our fermentations using LC-MS/MS.

Dide- and 14-deacetyl parasiticolide A were also found as genuine metabolites from the RIB40 strain and the compounds were present in multiple fermentations however, parasiticolide A was only detected in trace amounts using a LC-MS/MS method. This indicates a defective acetylation of the 14-deacetyl parasiticolide A and the small amount of parasiticolide A in RIB40 could be the result of spontaneous acetylation in the cell cytosol. The mono-deacetylated analogue detected in both *A. flavus* and *A. oryzae* had same retention times, suggesting a selective acetylation.

Altogether our findings contribute to understanding why the overall chemical profiles of *A. oryzae* and *A. flavus* appear quite different since some the end-products usually seen in *A. flavus* are

apparently not reached in *A. oryzae*. Whether the different chemical profiles are merely the result of different regulation that can be overcome by the use of epigenetic modifiers or are a result of genuine mutations remains to be settled.

*A. oryzae* RIB40 is clearly a chemical potent strain who live up to it variant name: *viridis*, and as more of its chemistry is unfolded we hypothesize that further biosynthetic pathways of *A. flavus* will be found to be more or less functional. It remains unanswered whether the aspirochlorine production in *A. oryzae*, which has not been reported from *A. flavus*, is unique for *A. oryzae* or just silent in *A. flavus*.

**Experimental Section**

**General experimental procedures**

General procedures and method for analysis is described in (Frisvad and Thrane, 1987; Smedsgaard, 1997; Frisvad et al., 2005).

Mass was measured using a Agilent HP 1100 liquid chromatograph with a DAD system (Waldbronn, Germany) on a LCT oaTOF mass spectrometer (Micromass, Manchester, UK) with a Z-spray ESI source and a LockSpray probe. For general procedures see (Nielsen et al., 2009); method 1 for LC-DAD-TOF was used in this study.

All solvents used were HPLC grade from Sigma-Aldrich (St. Louis, MO, USA).

**Fungal material and fermentation**

*A. oryzae* RIB40 (IBT28103); *A. flavus* NRRL3357 (IBT3696), (IBT15934), NRRL 13462; *A. parvisclerotigenus* IBT16807 and *A. minislcerotigenus* IBT13353 were obtained from the IBT Culture Collection at DTU Systems Biology, Technical University of Denmark.

The RIB40 isolate used for isolation of 13-dehydroxypaxilline was cultured for 14 days at 25°C in dark on 200 Petri dishes with Yeast Extract Sucrose agar (YES).

All strains were grown on for 1 week at 25°C on YES, Czapek Yeast Autolysate (CYA), Wickerhams Antibiotic Test Medium (WATM) agar (Raper and Thom, 1949), YESBEE (YES+50g Bee pollen Type III, granulate, Sigma, P-8753, pr. 1L medium), DRYES (Dichloran rose Bengal chloramphenicol agar), AFPA (*Aspergillus flavus*, *A. parasiticus* agar), CYAS (CYA+50g NaCl pr. 1L medium), CY20 (CYA+200g sucrose pr. 1L), CY40 (400g sucrose pr. 1L medium), DUL (Dulaney's medium for Penicillin), GAK (Potato-carrot agar), GMMS (Glucose minimal media (GMM) +2% sorbitol), MEA (Mout extract agar), OAT (Oat meal agar), PDA (Potato-dextrose agar). For medium formulations see Samson *et al.* (Samson et al., 2004)).

**Extraction and separation of 13-desoxypaxilline**

The plates were homogenized using a Stomacher and 100 mL EtOAc with 1% $HCO_2H$ pr. 10 plates. The extract was filtered and dried down on a freeze drier. The crude extract was separated on a KP-

C18-HS 60g SNAP column using a Biotage Isolera One (Biotage, Uppsala, Sweden), resulting in a 22mg fraction. The fraction was segmented with a 10g ISOL Diol column, using 12 steps of stepwise Heptane-dichloromethane-EtOAc-MeOH. 13-desoxypaxilline was predominant in a 100% EtOAc fraction (6mg), and purified on a Waters HPLC W600/996PDA (Milford, MA, USA) and a RP column (Phenomenex Luna C18(2), 250 x 10 mm, 5 µm, Torrance, CA, USA) using a gradient of 80% MeCN ($H_2O$ – Milli-Q (Millipore, MA, USA)) to 90% over 10 min. with 50ppm TFA added to the solvents. The collection was concentrated on a rotarvap (Büchi V-855/R-215, Flawil, Switzerland) and dried down under $N_{2\,(g)}$ to yield 0.5 mg of white, amorphous 13-desoxypaxilline.

**Isolation of di-, 14deactyl-, and 18-formyl parasiticolide A**

From the same fermentation described for 13-desoxypaxilline a more polar, 90mg fraction was fractionated with a 10g ISOL Diol column, using 12 steps of stepwise Heptane-dichloromethane-EtOAc-MeOH. The parasiticolide A-analogues were predominant in a 100% EtOAc fraction (10mg), and purified on a Waters HPLC W600/996PDA (Milford, MA, USA) and a RP column (Phenomenex Luna C18(2), 250 x 10 mm, 5 µm, Torrance, CA, USA) using a gradient of 72% MeCN ($H_2O$ – Milli-Q (Millipore, MA, USA)) to 87% over 15 min. with 50ppm TFA. The collection was concentrated on a rotarvap (Büchi V-855/R-215) and dried down under $N_{2\,(g)}$ to yield 0.3, 1.0 and 0.8 mg of white, amorphous di-, 14-deactyl-, and 18-formyl parasiticolide A, respectively.

**Selective extraction of sclerotia metabolites**

The selective extraction of sclerotia from IBT 15934, NRRL 13462, IBT 16807 and IBT 13353 was made from harvested sclerotia of a 7 day old cultivation on WATM and CYA agar (25°C in dark). The sclerotia were washed several times with Milli-Q (Millipore, Millford, USA) 0.22µm $H_2O$ and dried. The sclerotia were transferred to a 2 mL Eppendorf tube together with three stainsless steel balls (2*1mm and 1*5mm) and freezed with liquid $N_2$ before mechanical crushed. The pulverized sclerotia was suspended in 1mL methanol and transferred to a 2mL vial with 1mL of 1:2:3 methanol:dichloromethane:ethylacetate and left for evaporation over night in a fume hood. The dried extract was resolved in 1mL methanol in ultrasonicated for 10min and then filtered with a 0.45µm PTFE filter to a clean vial for analysis.

**MS/MS method used for aflatrem and parasiticolide screening**

Liquid chromatography was performed on an Agilent (Torrence, CA) 1100 HPLC system coupled to a triple-quadropole mass spectrometer (Waters-Micromass, Manchester, UK) with a Z-spray ESI operated in positive mode source using a flow of 700 L/h nitrogen desolvated at 350°C. Hexapole was held at 50V. The system was controlled by MassLynx v4.1 (Waters-Micromass). Nitrogen was used as collisioin gas, and the MS operated in MRM mode (dwell time = 100 ms) with the parameters listed in Table 2.

Extracts of 2µL were injected and separated on a Phenomenex Gemini $C_6$-phenyl, 3µm, 2*50mm column with a flow of 0.3µL/min. Water contained 20mM ammonium formate. Oven temperature 40.0°C. Two different methods were applied to the aflatrem- and parasiticolide screen:
Aflatrem inlet method: linearly gradient from 50 to 100% MeCN over 5min, increased to 0.5µL/min over 1.5 min. The column was washed additionally 1.5 min with 100% MeCN at 0.5µL/min, followed by a return to 50% MeCN over 2.5 min and kept at this level for another 1 min with a linearly decrease in flow to 0.3µL/min, prior to the next sample. Standards used for analysis of this pathway were from Sigma-Aldrich Aldrich (St. Louis, MO, USA).
Parasiticolides: linearly gradient from 20 to 90% MeCN over 15min, increased to 0.5µL/min from 90 to 100% MeCN in additional 1 min. The column was washed from 2 min with 100% MeCN at 0.5µL/min, followed by a return to 20% MeCN over 1.5 min and kept at this level for another 3.5 min with a linearly decrease in flow to 0.3µL/min, before the next sample. Standards were internal standards from other extracts of the known parasiticolide A producer *A. parasiticus*: IBT 4387 (CBS 260.67) and IBT 11863 (115.37).

**Table 2**. MS/MS method including scan event, retention times, transition ions and the cone and collision energies used.

| Compound | Scan event | RT/min | Ion type | Transition (*m/z*)[a] | Cone (V) | Collision energy (eV) |
|---|---|---|---|---|---|---|
| Paxilline | 1 | 4.0 | Quantifier | 436 → 130 | 25 | 30 |
| | | | Qualifier | 436 → 182 | 25 | 30 |
| Paspalinine | 2 | 4.3 | Quantifier | 434 → 130 | 25 | 20 |
| | | | Qualifier | 434 → 376 | 25 | 20 |
| 13-desoxypaxilline | 3 | 4.8 | Quantifier | 420 → 182 | 25 | 30 |
| | | | Qualifier | 420 → 130 | 25 | 30 |
| Aflatrem | 4 | 5.2 | Quantifier | 502 → 198 | 25 | 20 |
| | | | Qualifier | 502 → 445 | 25 | 20 |
| Paspaline | 5 | 5.5 | Quantifier | 422 → 130 | 25 | 20 |
| | | | Qualifier | 422 → 275 | 25 | 20 |
| Dideacetyl-parasiticolide A | 1 | 7.0 | Quantifier | 387 → 217 | 30 | 40 |
| | | | Qualifier | 387 → 189 | 30 | 40 |
| 14-deacetyl parasiticolide A | 2 | 8.7 | Quantifier | 429 → 217 | 30 | 40 |
| | | | Qualifier | 429 → 189 | 30 | 40 |
| Parasiticolide A | 3 | 10.4 | Quantifier | 488 → 229 | 30 | 30 |
| | | | Qualifier | 488 → 247 | 30 | 30 |

[a]All transitions were made from $[M+H]^+$, except for paraciticolide A: $[M+NH_4]^+$

**HPLC-DAD-TOF method**

Mass was measured using a Agilent HP 1100 liquid chromatograph with a DAD system (Waldbronn, Germany) on a LCT oaTOF mass spectrometer (Micromass, Manchester, UK) with a Z-spray ESI source and a LockSpray probe. For general procedures see (Nielsen et al., 2009), method 1.

**13-desoxypaxilline**

HRESIMS: m/z = 420.2551 $[M + H]^+$, calculated for $[C_{27}H_{33}NO_3+H]^+$: 420.2533.

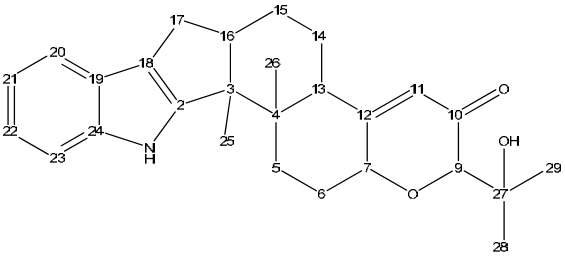
NMR spectra were acquired in DMSO-$d_6$ on a Bruker Avance 800 MHz spectrometer using standard pulse sequences. The spectra were referenced to this solvent with resonances $\delta_H$ = 2.50 and $\delta_C$ = 39.5. $^1$H NMR (799.30 MHz, DMSO-$d_6$, 25 °C, 2.50 ppm): 0.88 (3H, s, H-23), 1.00 (3H, s, H-25), 1.16 (3H, s, H-29), 1.20 (3H, s, H-28), 1.52 (1H, ddd, J = 15.4, 12.8, 4.4 Hz, H14a), 1.60 (1H, m, H-14b), 1.65 (1H, m, H-15a), 1.74 (1H, d, J = 12.2 Hz, H-15b), 1.81 (1H, ddd, J = 17.9, 13.8, 4.2 Hz, H-6a), 1.98 (1H, ddd, J = 13.8, 13.6, 4.2 Hz, H-5a), 2.07 (1H, m, H-5b), 2.22 (1H, m, H-6b), 2.32 (1H, dd, J = 12.8, 11.0 Hz, H-17a), 2.53 (1H, m, H-13), 2.62 (1H, dd, J = 12.8, 6.3 Hz, H-17b), 2.71 (1H, m, H-16), 3.74 (1H, d, J = 1.6 Hz, H-9), 4.34 (1H, br. s, 27-OH), 4.41 (1H, m, H-7), 5.73 (1H, s, H-11), 6.91 (1H, dd, J = 7.6, 7.6 Hz, H-21), 6.95 (1H, dd, J = 7.6, 7.6 Hz, H-22), 7.27 (1H, d, J = 7.6 Hz, H-23), 7.28 (1H, d, J = 7.6 Hz, H-20), 10.76 (1H, s, N-H).

$^{13}$C NMR (201.00, DMSO-$d_6$, 25 °C, 39.5 ppm): 14.4 (C-25), 15.4 (C-26), 23.5 (C-15), 24.8 (C-14), 25.5 (C-28), 25.7 (C-29), 26.7 (C-17), 29.5 (C-6), 30.7 (C-5), 41.6 (C-13), 48.5 (C-16), 49.2 (C-3), 49.8 (C-4),

70.7 (C-27), 74.0 (C-7), 82.4 (C-9), 111.6 (C-23), 115.8 (C-18), 117.5 (C-20), 118.1 (C-21), 119.2 (C-22), 120.7 (C-11), 124.4 (C-19), 140.2 (C-24), 150.4 (C-2), 168.6 (C-12), 196.1 (C-10).

References:

(Nozawa et al., 1988; 1989)



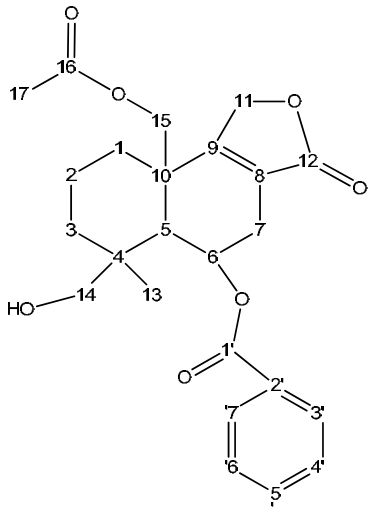
**14-deacetyl parasiticolide A**

All NMR spectra of parasiticolide A analogues were acquired in DMSO-$d_6$ on a Varian Unity Inova 500 MHz spectrometer with 4 mm gHX Nano probe and with a spin rate of 2 kHz for all samples, using standard pulse sequences. The spectra were referenced to this solvent with resonances $\delta_H$ = 2.50 and $\delta_C$ = 39.5.

HRESIMS: m/z = 429.1901 $[M + H]^+$, calculated for $[C_{24}H_{28}O_7+H]^+$: 429.1908.

$^1$H NMR (499.87 MHz, DMSO-$d_6$, 25 °C, 2.50 ppm): ): 0.96 (1H, td, J = 13.6, 3.7 Hz, H-3a), 1.01 (3H, s, H-13), 1.36 (1H, td, J = 13.3, 3.3 Hz, H-1a), 1.45 (1H, m, H-2a), 1.63 (1H, m, H-2b), 1.91 (1H, s, H-5), 1.97 (1H, m, H-3b), 2.08 (3H, s, H-17), 2.11 (1H, d, J = 13.2, H-1b), 2.36 (1H, d, J = 19.0 Hz, H-7a), 2.76 (1H, ddt, J = 19.0, 6.0, 3.1 Hz, H-7b), 3.14 (1H, d, J = 10.6, H-14a), 3.61 (1H, d, J = 10.6 Hz, H-14b), 4.67 (1H, d, J = 11.0 Hz, H-15a), 4.80 (1H, d, J = 11.0, H-15b), 4.96 (2H, m, H-11), 5.83 (1H, d, J = 6.0 Hz, H-6), 7.52 (2H, m, H-4'/6'), 7.67 (1H, tt, J = 7.3, 1.2 Hz, H-5'), 7.97 (2H, m, H-3'/7').

$^{13}$C NMR (125.70 MHz, DMSO-$d_6$, 25 °C, 39.5 ppm): 17.3 (C-2), 20.6 (C-17), 28.2 (C-7), 30.7 (C-1), 34.6 (C-3), 39.4 (C-4), 39.8 (C-10), 52.5 (C-5), 62.9 (C-14), 65.4 (C-15), 66.5 (C-6), 71.1 (C-11), 121.2 (C-8), 128.7 (4'/6'), 129.3 (3'/7'), 129.8 (C-2'), 133.5 (5'), 165.6 (C-1'), 166.8 (C-9), 170.5 (C-16), 173.2 (C-12).

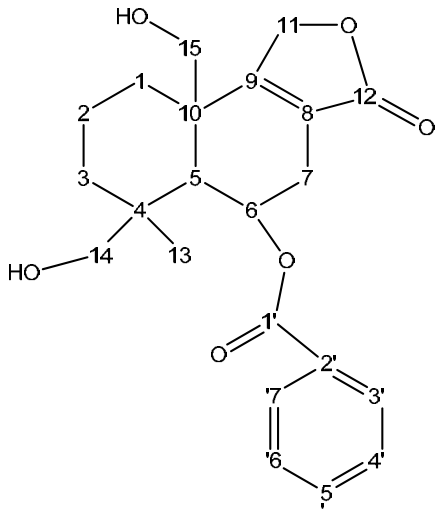


**Dideacetyl parasiticolide A**

HRESIMS: m/z = 387.1817 $[M + H]^+$, calculated for $[C_{22}H_{26}O_6+H]^+$: 387.1802.

$^1$H NMR (499.87 MHz, DMSO-$d_6$, 25 °C, 2.50 ppm): 0.91 (1H, ddd, J = 13.4, 13.4, 3.7 Hz, H-3a), 0.99 (3H, s, H-13), 1.17 (1H, dd, J = 12.9, 3.5 Hz, H-1a), 1.42 (1H, m, H-2a), 1.65 (1H, m, H-2b), 1.81 (1H, s, H-5), 1.97 (1H, d, J = 13.4 Hz, H-3b), 2.28 (1H, d, J = 12.9 Hz, H-1b), 2.31 (1H, d, J = 19.3 Hz, H-7a), 2.73 (1H, m, H-7b), 3.09 (1H, dd, J = 10.5, 5.3 Hz), 3.60 (1H, dd, J = 10.5, 5.3 Hz, H-14b), 4.07 (1H, m, H-15a), 4.12 (1H, m, H-15b), 4.42 (1H, dd, J = 5.3, 5.3 Hz, 14-OH), 4.86 (1H, dd, J = 17.5, 2.0 Hz, H-11a), 5.06 (1H, dt, J = 17.5 , 2.5 Hz, H-11b), 5.10 (1H, dd, J = 5.2, 5.2 Hz, 15-OH), 5.80 (1H, d, J = 5.8 Hz, H-6), 7.55 (2H, m, H-4'/6'), 7.67 (1H, m, H-5'), 7.90 (2H, m, H-3'/7').

$^{13}$C NMR (125.70 MHz, DMSO-$d_6$, 25 °C, 39.5 ppm): 17.6 (C-2), 27.3 (C-13), 28.5 (C-7), 30.5 (C-1), 34.9 (C-3), 39.4 (C-4), 42.9 (C-10), 52.7 (C-5), 62.4 (C-15), 63.1 (C-14), 66.9 (C-6), 72.0 (C-11), 119.5 (C-8), 128.9 (C-4'/6'), 129.0 (C-3'/7'), 129.8 (C-2'), 133.5 (C-5'), 165.7 (C-1'), 169.4 (C-9), 173.6 (C-12).

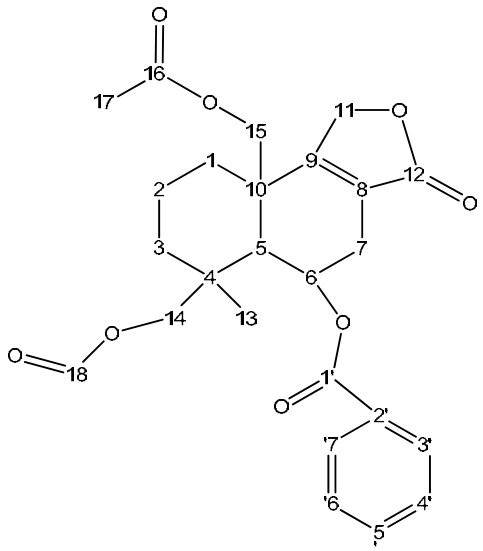


**18-formyl parasiticolide A**

HRESIMS: m/z = 457.1889 $[M + H]^+$, calculated for $[C_{25}H_{28}O_8+H]^+$: 457.1857.

$^1$H NMR (499.87 MHz, DMSO-$d_6$, 25 °C, 2.50 ppm): 1.10 (3H, s, H-13), 1.18 (1H, ddd, J = 13.7, 13.7, 3.3 Hz, H-3a), 1.43 (1H, ddd, J = 13.2, 13.2, 2.9 Hz, H-1a), 1.51 (1H, m, H-2a), 1.71 (1H, m, H-2b), 1.82 (1H, d, J = 13.7 Hz, H-3a), 2.06 (1H, s, H-5), 2.09 (3H, s, H-17), 2.16 (1H, d, J = 13.2 Hz, H-1b), 2.43 (1H, d, J = 19.3 Hz, H-7a), 2.80 (1H, m, H-7b), 3.99 (1H, d, J = 11.0 Hz, H-14a), 4.36 (1H, d, J = 11.0 Hz, H-14b), 4.71 (1H, d, J = 10.8 Hz, H-15a), 4.78 (1H, d, J = 10.8 Hz, H-15b), 4.98 (2H, m, H-11), 5.83 (1H, d, J = 5.6 Hz, H-6), 7.52 (2H, dd, J = 7.6, 7.6 Hz, H-4'/6'), 7.68 (1H, dd, J = 7.6, 7.6 Hz, H-5'), 7.98 (2H, d, J = 7.6 Hz, H-3'/7'), 8.06 (1H, s, H-18).

$^{13}$C NMR (125.70 MHz, DMSO-$d_6$, 25 °C, 39.5 ppm): 16.9 (C-2), 20.5 (C-17), 26.6 (C-13), 27.9 (C-7), 30.5 (C-1), 35.4 (C-3), 36.9 (C-4), 39.6 (C-10), 52.1 (C-5), 65.0 (C-14), 65.1 (C-15), 66.5 (C-6), 71.1 (C-11), 121.0 (C-8), 128.6 (C-4'/6'), 129.0 (C-3'/7'), 129.2 (C-2'), 133.4 (C-5'), 161.6 (C-18), 165.1 (C-1'), 165.9 (C-9), 170.1 (C-16), 172.8 (C-12).



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# 7 Comparative Metabolomics of *A. flavus* and *A. oryzae*: Unraveling the Chemical Diversity

This chapter is a presentation of a large effort to describe *A. flavus* chemistry holistically in a new and unproved manor. The analysis is aimed for publication in a peer-reviewed journal, but here the work is presented as one story describing *A. flavus* metabolomics. The authors involved in the article are: Rank, C.[a], Hansen, M.A.E.[b], Larsen, T.O.[a] and Frisvad, J.C.[a]

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## Introduction

*Aspergillus flavus* is a diverse species in many respects: the genetic variability is large enough to allow for "sub-speciation", the morphological differences have troubled mycologists and others for decades and still poses notorious difficulties in separating *A. flavus sensu stricto* from the broader definition (*sensu lato*) and this is also reflected in the chemodiversity of these fungi. This genetic and mycotoxic diversity is well established in many studies, however the full chemical potential of these fungi has not been globally analyzed and reviewed in comparison to the knowledge of former studies. Many compounds have been isolated in the quest for uncovering more of the truth behind the "Turkey X" disease (Blount, 1961; Spensley, 1963) – many via toxicity assays – and particularly aflatoxins and their precursors have gained much attention. The aflatoxins definitely shifted the focus to secondary metabolite research. This inevitably left many metabolites undiscovered, but in recent years genome sequencing has prompted for a full mapping of all potential secondary metabolites in both genome and transcriptome studies and has paved the way for secondary metabolomic studies (Rank et al., 2010).

The diversity of *Aspergillus* section *Flavi* is an important parameter for the understanding of genome sequencing, chemistry and ecology, as these species are the most important producers of aflatoxin and many other bioactive secondary metabolites.

The diversity have been analyzed with various genetic tools, vegetative compatibility groups (VCGs), morphological examinations and mycotoxin analysis.

## Genetics and Diversity

Genetic work on *Aspergillus* section *Flavi* has been carried out for decades and has resulted in a better understanding of the toxigenic aspects of *A. flavus*, though many of the ecological facets have yet to be established.

The species characteristica of *A. flavus* have been studied extensively and subsequently the species description has been revised several times (Christensen, 1981; Egel et al., 1994; Geiser et al., 2000).

Generally *A. flavus* can be divided into two groups depending on the average size the sclerotia: L-type for sclerotium >400µm and S-type for sclerotia <400µm (Cotty, 1989). The L-type produce abundant conidiospores and fewer sclerotia, while the S-type display the opposite signature with often extreme sclerotia production and few conidiospores. This rough separation of *A. flavus* into L- and S-sclerotia types has been recognized for many years. Bayman and Cotty (Bayman and Cotty, 1993) suggested that they were two genetically distinct groups, which was later confirmed by Geiser et al. (Geiser et al., 1998; Geiser et al., 2000). The enormous genetic diversity in section *Flavi* is well documented in the many VCGs found for *A. flavus* both for the genuine L-sclerotial isolates and the S-type (Bayman and Cotty, 1991; 1993; Horn and Greene, 1995). The existence of the many VCGs and the recent discovery of *MAT1-1* and *MAT1-2* mating-type genes in *A. flavus* by Ramirez-Prado et al. (Ramirez-Prado et al., 2008) have led to the intricate, but successful attempts with two sexually compatible *A. flavus* strains to mate and produce stromata, resulting in the teleomorph *Petromyces flavus* (Horn et al., 2009a). The effect on metabolite production was not investigated further. This possible perfect state of *A. flavus* offers some explanation to the large genetic diversity; inconsistent with a purely clonal population.

Differences in aflatoxin production is also coupled to the two types of *A. flavus*, as noted by Hesseltine in 1970 (Hesseltine et al., 1970) and Saito (Saito et al., 1986; 1989; Saito and Tsuruta, 1993): the small sclerotial isolates produce aflatoxin B and G in high amounts, whereas the large sclerotium producer only synthesize aflatoxin B or none at all (Cotty, 1997). The L-type is identical with *A. flavus sensu stricto* exemplified with the non-aflatoxigenic type culture (NRRL 1957) and aflatoxigenic isolate chosen for genome sequencing (NRRL 3357). The S-type has been divided into *A. minisclerotigenes* (Pildain et al., 2008) – the study includes four of the S-types *A. flavus* isolates used by (Geiser et al., 1998) – and *A. parvisclerotigenus* (Saito and Tsuruta, 1993; Frisvad et al., 2005). It is not unanimously recognized that these two species engulf all biodiversity of the S-types (Atehrikeng et al., 2008), but the number of available strains of these types are much lower than for *A. flavus*. *A. parasiticus* produce L-type sclerotia, but is recognized as a different species and it produces aflatoxin more consistently including aflatoxin G, which is not in line with the perception of *A. flavus sensu stricto*. The perfect state of *A. parasiticus* (*Petromyces parasiticus*) was also found recently by Horn et al. (Horn et al., 2009b). *P. alliaceus* is the third member of section *Flavi* with a known sexual state.
Studies of the aflatoxin gene clusters of the L-type *A. flavus* have resulted in the discovery of at least two types of either 0.8-1.0 kb or 1.5 kB deletion in the *norB*/*cypA* genes responsible for conversion of aflatoxin B to G (Ehrlich et al., 2004; Chang et al., 2006).

Of the many studies dealing with *Aspergillus* genetics, especially the nature of the difference between *A. flavus* and *A. oryzae* has attracted much of the attention. With the recent genome sequencing of both species (Machida et al., 2005; Payne et al., 2007; Cleveland et al., 2009), many of

the early studies have been confirmed and new details are uncovered at an accelerated pace and the first whole genome comparisons have validated the many published indications for the tight relationship between the two species. If *A. oryzae* was not used widely in industry it would probably not have maintained its species recognition (Geiser et al., 1998; 2000; Chang et al., 2006; Payne et al., 2006; 2007; Machida et al., 2008). *A. oryzae* is perceived as the domesticated form of *A. flavus* (Geiser et al., 2000; Rokas, 2009).

Despite the enormous research efforts to ensure the safety of *A. oryzae*, most of the predicted genes for secondary metabolites have yet to be coupled to existent metabolites (Yu et al., 2008). Bioinformatic studies in concurrence with the genome sequencing shows approximately the same number of predicted genes for these two species: 32 polyketide synthases (PKSs) and 28 non-ribosomal synthases (NRPSs) for *A. flavus* and 32 PKSs and 27 NRPSs for *A. oryzae* with 2 NRPSs apparently unique for each strain (Cleveland et al., 2009). The exclusiveness of these genes in terms of end product has yet to be verified chemically. The question is how many metabolites these genes encode in amongst 77 ABC transporters and more than 122 cytochrome p450 enzymes predicted (Payne et al., 2007). Today only the genes for the important toxins have been fully annotated: aflatoxin (Yu et al., 2004), cyclopiazonic acid (Tokuoka et al., 2008; Chang et al., 2009a) and aflatrem (Nicholson et al., 2009). Other known metabolites, such as asperfuran, aflavarin, aflavinines and aflavazole, maltoryzine, aspirochlorine and the β-nitropropionic acid has yet to be mapped to the genome of *A. flavus*/*A. oryzae*, and though prominent compounds, they only represent a small part of the full chemical potential of these fungi.
The aflatoxin gene cluster has been studied in depth for *A. flavus* and *A. parasiticus*, but also in *A. oryzae* with the purpose of understanding the non-aflatoxicity of this domesticated *A. flavus* (Chang et al., 2006). Research indicates the discovery of minor mutations and truncations, especially in the aflatoxin regulating *aflR*, as explanation for the apparently dysfunctional gene cluster (Yu et al., 2008). The cyclopiazonic acid (CPA) gene cluster has been found adjacent to the aflatoxin cluster on chromosome 3 (Tokuoka et al., 2008; Chang et al., 2009a), and the aflatrem biosynthesis has recently been studied in *A. flavus* and *A. oryzae* and compared to *P. paxilii*, which produces the aflatrem precursor paxilline (Nicholson et al., 2009). Unlike the consensus for most fungal secondary biosynthetic genes, the genes for aflatrem are not clustered together, but separated in two clusters on chromosome 5 (*ATM1*) and 7 (*ATM2*). Both aflatoxin, CPA and aflatrem are regulated on the transcription level via the sclerotia correlated *veA* in *A. flavus* (Cary et al., 2007; Duran et al., 2007), a protein shown to be part of the velvet complex, VelB/VeA/LeaA, in *E. nidulans* and important for the regulation of sexual reproduction and secondary metabolite production as a function of light (Bayram et al., 2008; Calvo, 2008). Homologue sequences of the mating gene *MAT1-1* were also found in *A. oryzae*, which might pose a security threat in industry applications (Ramirez-Prado et al., 2008).

The genetic and mycotoxigenic variances have been coupled to field studies of sampled fungi from around the world, with a relative clear separation of the species into ecological niches: *A. flavus* is predominantly isolated from corn and peanut fields, often invading the crops, whereas *A. parasiticus*, *A. nomius*, *A. bombycis*, *A. toxicarius* and the group of small-sclerotium producers; *A. minisclerotigens* and *A. parvisclerotigens* are found more often in soil of these fields as well as outside, on peanuts or on insects. *A. flavus* has a much wider distribution compared to its more toxigenic relatives and may have a general advantage with an adaptation to the semi-controlled environment in corn-fields in particular (Horn and Greene, 1995; 1999; Cardwell and Cotty, 2002; Jaime-Garcia and Cotty, 2006a; 2006b; Horn, 2007). The aflatoxin precursor sterigmatocystin, which is found in many different genera [Rank et al. 2009b, in prep], has been shown to be important in the sporulation of *Emericella nidulans* and potentially important for fungal survival (Wilkinson et al., 2004). Cotty investigated the distribution of L- and S-type *A. flavus* in cotton fields in Arizona, US and found that the S-type did produce much higher amounts of aflatoxin *in vitro*, but *A. flavus* (L-type) was more pathogenic towards cotton plants *in vivo* as the devastation caused in cotton balls was greater. Neither showed a positive correlation between aflatoxin levels and pathogenic behavior, however a non-aflatoxigenic isolate showed aggressive behavior towards the cotton plants (Cotty, 1989). Interestingly, CPA appears to be much more consistently expressed in *A. flavus*, even in non-aflatoxigenic isolates and *A. oryzae* and might have a more important function in the fungal defense than has previously been recognized. The tetrameric acid CPA is known as a strong metal chelator and this fundamental function might be an explanation for the consistent production. Air samples from corn fields also indicate how *A. flavus* thrives above ground, which would inevitably aid the dispersal of the *A. flavus* spores (Hill et al., 1984).

In undeveloped countries where hunger is a genuine problem, many may be forced to eat infested crops, such as corn. It has been shown that up till 99% of the population in certain parts of West African countries have been exposed to aflatoxin contamination (Gong et al., 2002). One method that has been tried *in vivo* is biocontrol, using non-aflatoxigenic strains of *A. flavus* (Cotty, 1994). The potential of recombination with toxigenic isolates has recently been discussed by (Moore et al., 2009).

### Expression of Chemical Potential

An essential obstacle to overcome besides precise annotation is the *in vitro* expression of this potential. Many gene clusters appear to be silenced, thus revealing no products under normal growth conditions (Bok et al., 2006; 2009; Williams et al., 2008; Bergmann et al., 2007), which makes it difficult to assess the important, putatively assigned secondary metabolites genes. The task of molecular genetic modification to express each potential gene is cumbersome, especially if more clusters are to be found divided as with the discovery of the separated gene cluster of aflatrem in *A. flavus/A. oryzae*. The discovery of histone inhibiting molecules that modifies the DNA transcription at

an epigenetic level offers one prospective approach, but research on what metabolites triggers this DNA unfolding is still young and the mechanisms not fully understood (Shwab et al., 2007; 2008; Williams et al., 2008; Bok et al., 2009). The aflatoxins are some of the best investigated metabolites in terms of the producing fungi's optimal growth conditions, where temperature, water activity, pH and substrate composition play an important role for the yields (Luchese and Harrigan, 1993; Molina and Giannuzzi, 2002; Giorni et al., 2008; Schmidt-Heydt et al., 2009), but this knowledge is not applied to most of the remaining metabolites. Another, more readily approachable solution is to use different complex media that might themselves contain epigenetic modifying compounds and via multiple cultivations detect the chemo diversity and compare with the number of bioinformatically predicted products. This approach has been formalized as OSMAC – One Strain Many Compounds by Bode et al. (Bode et al., 2002). Neither of these techniques or approaches ensures that the synthesized compounds are produced in sufficient levels to allow for identification or structure elucidation. The complex regulation of the full set of secondary metabolites is not fully understood and feedback regulation could be one factor limiting for products to surface to the identification level.

The use of many different isolates of one species could aid to cover the chemical space in a different fashion, as this current data set clearly presents. Complementary to OSMAC, one could call this OCMAS – One Compound Many Strains, and though it appears awkward at first, this may prove important to unveil all metabolites when bioinformatic predictions are vague and imprecise. In this study the genome sequenced isolates *A. flavus* NRRL 3357 and *A. oryzae* RIB40 do not produce all the chemistry presented by a large section of strains and even with the right OSMAC setup or all necessary epigenetic regulating metabolites, some secondary metabolite genes may be nonfunctional, thus not presenting the genomic full potential of a given species. In this study, a new metabolite for *A. flavus* is reported from the non-aflatoxigenic ex type culture (NRRL 1957), but this metabolite remains non-detectable on many media in the toxigenic *A. flavus* NRRL 3357.

To fully understand and describe a given species, enough strains must be selected for OSMAC cultivation, which represents the entire chemodiversity. The chemical annotation of all metabolites and separation into biosynthetic families, will give a more complete picture of the chemical potential based on active biosynthetic pathways and not single metabolites, which is merely an expression of chemo diversity, and the backwards mapping of secondary metabolite genes, will then be possible. And as Kersten and Dorrestein argued, a full elucidation of the secondary metabolome is important for the understanding of the *in vivo* function and the correlation to the global regulation (Kersten and Dorrestein, 2009).

## Metabolomics

Research at the metabolomic level of *A. flavus* and *A. oryzae* has primarily focused on the mycotoxigenic aspects of these fungi; either as crop and food contamination of *A. flavus* or for the toxigenic potential of the "generally regarded as safe" species *A. oryzae* (Blumenthal, 2004).

Metabolomic studies, such as defined by Fiehn (Fiehn, 2002) with full insight on all possible metabolites, have never been carried out for these fungi; only analysis of selected toxin levels and distribution.

Studies have previously noted the genetic and chemical diversity within *A. flavus*; especially the great variations in production of aflatoxin B with up till 70% are non-alfatoxigenic in some areas (Bayman and Cotty, 1991; 1993; Cotty, 1989; 1997; 1999; Jaime-Garcia and Cotty, 2006a).

The divergence in aflatoxin production has received much focus in *A. flavus* research, but besides some spatial co-studies of the distribution of CPA and β-nitropropionic acid, no efforts have been made to assess the remaining mycotoxigenic potential of *A. flavus* field isolates. Cyclopiazonic acid has been proposed as a co-factor in the Turkey X disease by Cole (Cole, 1986). Production of cyclopiazonic acid, β-nitropropionic acid and kojic acid is shared with the domesticated form *A. oryzae*, but it has been shown that CPA is degraded in *A. oryzae* fermentations (Tanaka et al., 2002). The aflatoxin variation might be a feature that extends beyond these important toxins, to the less prominent chemical constituents of *A. flavus* and *A. oryzae*, with important implications for those parts of the world, where consumption of available food is a necessity, regardless of the risks as well as for the biocontrol with nonaflatoxigenic *A. flavus* strains. So as more biosynthetic routes are mapped, toxigenic studies of the remaining (majority) of metabolites should also be undertaken.

One of the essential questions that needs answering is what happens in the transition from the pathogenic wild type *A. flavus*, found on insects, cotton, nuts and crops to the domesticated, non-pathogenic, non-aflatoxigenic *A. oryzae*, used in fermentations of foods and production of pharmaceuticals. The toxicity of *A. oryzae* metabolites is of great importance for industry and has been extensively researched as indicated; see reviews (Barbesgaard et al., 1992; Burdock and Flamm, 2000; 2001; Blumenthal, 2004). Sterigmatocystin and aflatoxins have never been detected in *A. oryzae* (or in *A. sojae*, which is the domesticated form of *A. parasiticus*), and this correlates with genetic evidence of disrupted aflatoxin genecluster (Chang et al., 2007; Yu et al., 2008).

In terms of chemical insight, only loss of aflatoxin production has been recognized as a true metabolic change from *A. flavus* to *A. oryzae*; however several metabolites have been isolated from the domesticated *A. oryzae* which have never been reported from *A. flavus*, which might also be part of the metabolic shift. The two species have 99.5% gene homology, the same order of intraspecies differences between the two *A. fumigatus* sequenced strains as well as the two *A. niger* isolates sequenced, emphasizing their close relation (Rokas et al., 2007). The same article argues that there are two unique non-ribosomal peptide synthases in each species. A more careful dissection of this

aspect has been left mainly untouched, however it is important for the holistic perception and understanding of *A. flavus* to express and characterize these metabolites. In this study we apply a new approach to capture both the essence and diversity of *A. flavus*/*A. oryzae*.

**Known and important metabolites from *Aspergillus* section *Flavi***

For a holistic, chemical analysis of *A. flavus* and *A. oryzae* the related, more toxic species needs to be included in order to understand the distribution of the different metabolites and the potential implications for the yin/yang *A. oryzae*/*A. flavus*.

In the article by Pildain et al. (Pildain et al., 2008), two new aflatoxin producers were reported: *Aspergillus arachidicola* and *A. minisclerotigenes* and the chemistry of these species along with a selection of *Aspergillus* section *Flavi* members assessed. In Table 1 we list some of the most important (chemotaxonomically and toxic) metabolites for *Aspergillus* section *Flavi*.

**Table 1.** Metabolite production of *Aspergillus* section *Flavi* members, adapted from (Frisvad et al., 2005; Pildain et al., 2008) and expanded.

| | Sclerotium production and size | Kojic acid | Aflatoxin $B_1$, $B_2$ | Aflatoxin $G_1$,$G_2$ | Cyclopiazonic acid | Aspergillic acid | Asperfuran | Parasiticolides | Aflavarins | Paspalinine/ paspaline/ aflatrem | Aflavinines | Nominine |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *A. arachidicola* | - | + | + | + | - | + | - | + | - | - | - | - |
| *A. bombycis* | - | + | + | + | - | ±[a] | - | - | - | - | - | - |
| *A. caelatus* | - | + | - | - | + | - | - | - | - | - | - | - |
| *A. flavus* | L | + | ±[a] | - | ±[a] | + | ±[a] | ±[a] | - | ±[a] | - | - |
| *A. minisclerotigenes* | S | + | + | + | + | + | - | + | + | + | + | - |
| *A. nomius* | - | + | + | + | - | + | - | - | - | - | - | + |
| *A. oryzae* | L | + | - | - | ±[a] | ±[a] | ±[a] | ±[a] | - | ±[a] | ±[a] | - |
| *A. parasiticus* | L | + | + | + | - | + | - | + | - | ±[a] | - | - |
| *A. parvisclerotigenus* | S | + | + | + | + | + | - | - | + | + | + | - |
| *A. pseudotamarii* | - | + | + | - | ±[a] | - | - | - | - | - | - | - |
| *A. sojae* | - | + | - | - | - | + | + | - | - | - | - | - |
| *A. tamarii* | - | + | - | - | ±[a] | - | - | - | - | - | - | - |
| *A. terricola* | - | + | - | - | - | - | - | - | - | - | - | - |
| *A. toxicarius* | - | + | + | + | - | + | - | + | - | - | - | - |

[a]Inconsistent production of the metabolite.

The overview in Table 1 of the typical metabolite from members of *Aspergillus* section *Flavi* presents the main metabolite similarity as being kojic acid. Most of the species are aflatoxigenic and produce both aflatoxin $B_1$ and $G_1$, with the exception of the non-aflatoxigenic *A. caelatus* and *A. tamarii*. *A. flavus* is an inconsistent producer of aflatoxin B as discussed earlier. *A. minisclerotigenes* and *A. parvisclerotigenus* and partly *A. bombycis* and *A. nomius* are all strong aflatoxin producers, and the

two former species produce aflatoxins as the most abundant metabolites. The primary differences between the two small

sclerotium producing species, *A. minisclerotigenes* and *A. parvisclerotigenus*, in terms of extrolite profiles was the production of parasiticolide in the former species and aspirochlorine in the latter species.

Interestingly aspirochlorine (=oryzachlorine) was also reported along with parasiticolide, and two unidentified, tentatively labeled "NO2" and "EPIF" from *A. arachidicola* (Pildain et al., 2008). *A. flavus* isolates in the Pildain study also displayed production of aspirochlorine and unknown "flavimine". Klausmeyer et al. found aspirochlorine and a tri- and tetrathio analogue along with the diketopiperazine cyclo(L-Leu-L-Trp) in an *A. flavus* isolate (Klausmeyer et al., 2005).

Parasiticolides do occur in *A. flavus*, though they have been reported once in literature (Shiomi et al., 2002), and in our studies we rarely saw any traces in HPLC-UV/Vis diode array detection (DAD) analysis.

A truly exhausting list of true *A. flavus* metabolites reported from literature is difficult to assure because of the many misidentifications of isolates and the questionable isolation and chemistry of some early papers. A none-validated list of likely metabolites can however be used for de-replication purposes in the quest of metabolite scavenging. In Table 2 we list those metabolites we believe are true *A. flavus* and *A. oryzae* metabolites and which have been used for model-improvement in the multivariate analysis of the generated DIMS data presented in this paper.

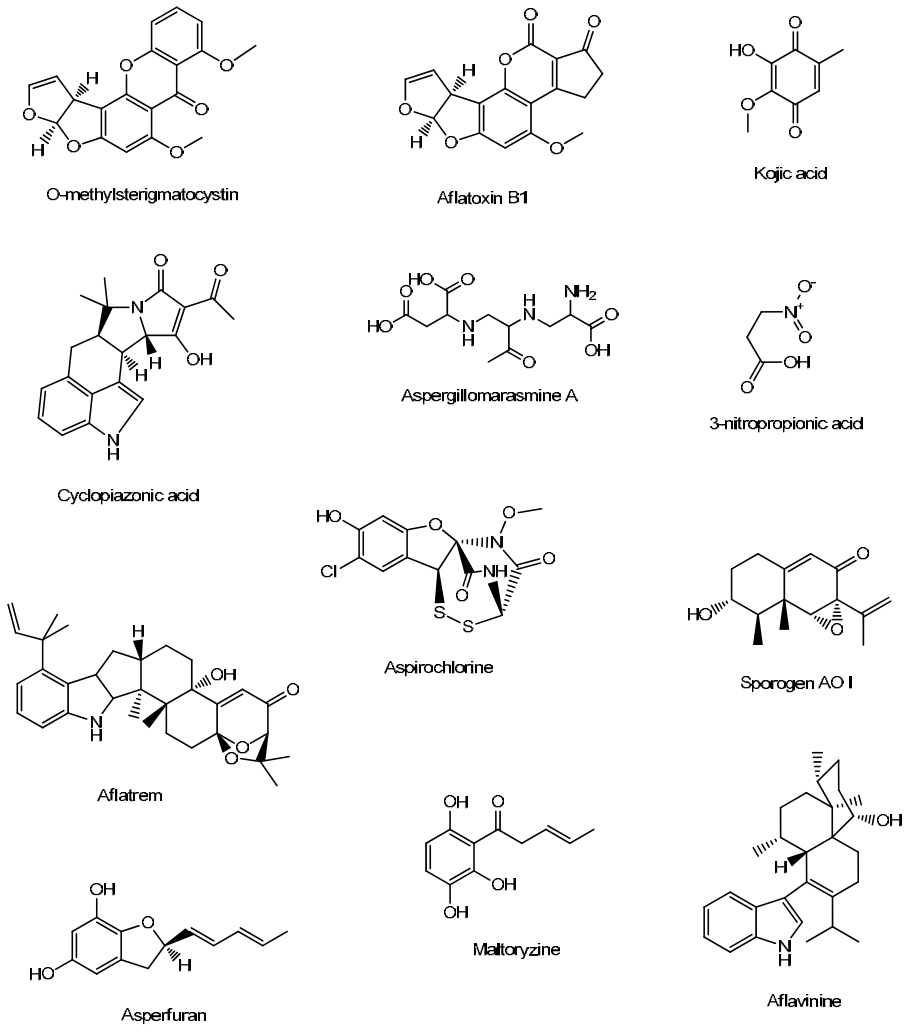


**Figure 1**. Important metabolites of *Aspergillus flavus* and *A. oryzae*.

**Table 2**. Known metabolites from *A. flavus* (L-type) and *A. oryzae* reported in literature[a].

| Metabolite | Molecular formula | *A. flavus* reference | *A. oryzae* reference |
|---|---|---|---|
| **Aflatoxin family** | | | |
| Aflatoxin $B_1$ | $C_{17}H_{12}O_6$ | (Asao et al., 1963; Hartley et al., 1963; Van der Merwe et al., 1963) | |
| Aflatoxin $B_2$ | $C_{17}H_{14}O_6$ | (Hartley et al., 1963; van Dorp et al., 1963) | |
| Aflatoxin $B_3$ (= parasiticol) | $C_{16}H_{14}O_6$ | (Heathcote and Dutton, 1969) | |
| Aflatoxin $B_{2\alpha}$ | $C_{17}H_{14}O_7$ | (Dutton and Heathcot, 1968) | |
| Aflatoxin $M_1$ | $C_{17}H_{12}O_7$ | (Dutton et al., 1985) | |
| Aflatoxin $M_2$ | $C_{17}H_{14}O_7$ | (Dutton et al., 1985) | |
| Aspertoxin | $C_{19}H_{14}O_7$ | (Rodricks et al., 1968; Waiss et al., 1968) | |
| O-methylsterigmatocystin | $C_{19}H_{14}O_6$ | (Burkhard and Forgacs, 1968) | |
| Sterigmatocystin | $C_{18}H_{12}O_6$ | (Schroeder and Kelton, 1975) | |
| Asparasone A | $C_{18}H_{14}O_8$ | Rank et al., this paper | |
| **Aflatrem family** | | | |
| Aflatrem (=a,a-Dimethylallylpaspalinine) | $C_{32}H_{39}NO_4$ | (Gallagher and Wilson, 1978; Gallagher et al., 1980a; Wicklow and Cole, 1982; Cole et al., 1981) | |
| β-aflatrem | $C_{32}H_{39}NO_4$ | (Tepaske et al., 1992) | |
| 13-desoxypaxilline | $C_{27}H_{33}NO_3$ | | Rank et al., 2009, in prep |
| Paspaline | $C_{28}H_{39}NO_2$ | | Rank et al., 2009, in prep |
| Paspalicine | $C_{27}H_{31}NO_3$ | | |
| Paspalinine | $C_{27}H_{31}NO_4$ | (Cole et al., 1981) | |
| **Aflavinine family** | | | Rank et al., 2009, in prep |
| Aflavazole | $C_{28}H_{35}NO_2$ | (Tepaske et al., 1990) | |
| Aflavinine | $C_{28}H_{39}NO$ | (Gallagher et al., 1980b; Nozawa et al., 1989) S-type | |
| 24,25-dehydro-10,11-dihydro-20-hydroxyaflavinine | $C_{28}H_{39}NO_2$ | (Gloer et al., 1988) | |
| 10,11-dihydro-11,12-dehydro-20-hydroxyaflavinine | $C_{28}H_{39}NO_2$ | (Gloer et al., 1988) | |
| 20,25-dihydroxyaflavinine | $C_{28}H_{39}NO_3$ | (Gloer et al., 1988) | |
| 20,26-Dihydroxyaflavinine | $C_{28}H_{39}NO_3$ | (Cole et al., 1981) S-type? NRRL 3251 | |
| 20-Hydroxyaflavinine (Monohydroxyaflavinine) | $C_{28}H_{39}NO_2$ | (Gloer et al., 1988) | |
| Monohydroxyisoaflavinine | $C_{28}H_{39}NO_2$ | (Nozawa et al., 1989) S-type | |
| **Kotanin family** | | | |
| Aflavarin | $C_{24}H_{22}O_9$ | (Tepaske et al., 1992) | |
| Kotanin | $C_{24}H_{22}O_8$ | (Tepaske et al., 1992) | |
| Demethylkotanin | $C_{23}H_{20}O_8$ | (Tepaske et al., 1992) | |
| **Aspirochlorine family** | | | |
| Aspirochlorine = oryzachlorine (=A30641) | $C_{12}H_9ClN_2O_5S_2$ | (Klausmeyer et al., 2005; Sakata et al., 1982; Sakata et al., 1987) | (Kato et al., 1969; Sakata et al., 1987; Sakata et al., 1983) |
| Trithioaspirochlorine | $C_{12}H_9ClN_2O_5S_3$ | (Klausmeyer et al., 2005) | |
| Tetrathioaspirochlorine | $C_{12}H_9ClN_2O_5S_4$ | (Klausmeyer et al., 2005) | |
| **Cyclopiazonic acid family** | | | |
| α-Cyclopiazonic acid | $C_{20}H_{20}N_2O_3$ | (Luk et al., 1977; Gallagher et al., 1978; Richard et al., 1992; Gqaleni et | (Matsudo and Sasaki, 1995; Orth, 1977; Chang et al., 2009b) |

| | | | |
|---|---|---|---|
| | | al., 1996; Horn and Dorner, 1999; Vinokurova et al., 2007; Chang et al., 2009b) | |
| β-Cyclopiazonic acid | $C_{20}H_{22}N_2O_3$ | (Chang et al., 2009b) | (Chang et al., 2009b) |
| Speradine A | $C_{21}H_{22}N_2O_4$ | Rank et al., this paper | |
| Cyclopiamine | $C_{26}H_{33}N_3O_5$ | (Bond et al., 1979) – Ref. not *A. flavus*, Rank et al., this paper, tentative HPLC-UV | |
| **Other tryptophan derived metabolites** | | | |
| Ditryptophenaline | $C_{42}H_{40}N_6O_4$ | (Springer et al., 1977) | |
| Cyclo(D-N-methyl-Leu-L-Trp) | $C_{18}H_{23}N_3O_2$ | (Klausmeyer et al., 2005) | |
| Miakamides $A_1$ | $C_{31}H_{32}N_4O_3$ | (Shiomi et al., 2002) | |
| Miyakamide $A_2$ | $C_{31}H_{32}N_4O_3$ | (Shiomi et al., 2002) | |
| Miyakamide $B_1$ | $C_{31}H_{32}N_4O_4$ | (Shiomi et al., 2002) | |
| Miyakamide $B_2$ | $C_{31}H_{32}N_4O_4$ | (Shiomi et al., 2002) | |
| **Parasiticolide family** | | | |
| Parasiticolide A = astelloide A | $C_{26}H_{30}O_8$ | (Shiomi et al., 2002) | Rank et al., 2009, in prep |
| Mono-deacetyl parasiticolide A | $C_{24}H_{28}O_7$ | | Rank et al., 2009, in prep |
| Di-deacetyl parasiticolide A | $C_{22}H_{26}O_6$ | | Rank et al., 2009, in prep |
| Sporogen AO1 | $C_{15}H_{20}O_3$ | | (Tanaka et al., 1984a; 1984b) |
| **Benzoderivatives** | | | |
| Asperfuran = arthrographol | $C_{13}H_{14}O_3$ | | (Pfefferle et al., 1990) |
| Maltoryzin | $C_{11}H_{12}O_4$ | | (Iizuka and Iida, 1962) (var. *microsporis*) |
| **Vitamines** | | | |
| Vitamine $B_1$ (Thiamine) | $C_{12}H_{17}N_4OS$ | (Srinivasan and Ramakrishnan, 1952) | (Fukui et al., 1955) |
| Vitamine $B_2$ (Riboflavin - Flavin pigment) | $C_{17}H_{20}N_4O_6$ | (Pontovich, 1943; Zalesskaya, 1950) | (Mogi et al., 1951; 1952; Higuchi, 1956) |
| Vitamine $B_5$ (Pantothenic acid) | $C_9H_{17}NO_5$ | | (Fukui et al., 1955) |
| Vitamine $B_{12}$ (Cyanocobalamine) | $C_{63}H_{89}CoN_{14}O_{14}P$ | | (Fukui et al., 1955) |
| **Nucleotide derivatives** | | | |
| Asperopterin A | $C_{13}H_{17}O_7N_5$ | | (Matsuura et al., 1972) |
| Asperopterin B | $C_8H_9N_5O_3$ | | (Matsuura et al., 1972) |
| **Kojic acid** | | | Review: (Bentley, 2006) |
| Kojic acid | $C_6H_6O_4$ | (Birkinshaw et al., 1931; Shiomi et al., 2002) | (Birkinshaw et al., 1931; Saito, 1907; Yabuta, 1912; 1913; 1916) |
| Kojic acid dimer | $C_{12}H_{10}O_8$ | (Zeringue et al., 1999) | |
| **Aspergillic acid family** | | | |
| Aspergillic acid | $C_{12}H_{20}N_2O_2$ | (Waksman and Bugie, 1943; White and Hill, 1943; Woodward, 1947; Dutcher, 1958; Macdonald, 1973) | |
| 1-Hydroxy-6-(1-methylethyl)-3-(2-methylpropyl)pyrazin-2-one | $C_{11}H_{18}N_2O_2$ | (Macdonald, 1973) | |
| Flavacol (= deoxyaspergillic acid?) | $C_{12}H_{20}N_2O$ | (Dunn et al., 1949) | |
| Hydroxyaspergillic acid | $C_{12}H_{20}N_2O_3$ | (Macdonald, 1973; Shiomi et al., 2002) | |
| Mutaaspergillic acid | $C_{11}H_{18}N_2O_3$ | (Macdonald, 1973) | |
| Neoaspergillic acid | $C_{12}H_{20}N_2O_2$ | (Macdonald, 1973) | |
| Isoflavipucine | $C_{12}H_{15}NO_4$ | | |

| | | | |
|---|---|---|---|
| Omoflavipucine | $C_{13}H_{17}NO_4$ | (Casinovi et al., 1980) | |
| **Other simple acids** | | | |
| Aspergillomarasmine A (=lycomarasmin from *Fusarium*) | $C_{10}H_{17}N_3O_8$ | | (Robert et al., 1962; Haenni et al., 1965) |
| Anhydroaspergillomarasmine B | $C_9H_{12}N_2O_7$ | | (Robert et al., 1962; Haenni et al., 1965) |
| Citric acid | $C_6H_8O_7$ | (Sakaguchi et al., 1953) | |
| Flufuran | $C_6H_6O_4$ | (Evidente et al., 2009) | |
| Fumaric acid | $C_4H_4O_4$ | (Sakaguchi et al., 1953) | |
| α-ketoglutaric acid | $C_5H_6O_5$ | (Sakaguchi et al., 1953) | |
| Kojistatin A | $C_{17}H_{32}N_4O_5$ | | (Sato et al., 1996) |
| Lactic acid | $C_3H_6O_3$ | | (Sakaguchi et al., 1953) |
| *l*-Malic acid | $C_4H_6O_5$ | | (Sakaguchi et al., 1953) |
| 3-nitropropionic acid = β-nitropropionic acid = Oryzacidin | $C_3H_5NO_4$ | (Bush, 1943; 1945; 1951; Iwasaki and Kosikowski, 1973) | (Nakamura and Shimoda, 1954; Iwasaki and Kosikowski, 1973) |
| Stachydrine (proline betaine) | $C_7H_{13}NO_2$ | | (Takata, 1929) |
| Succinic acid | $C_4H_6O_4$ | | (Sakaguchi et al., 1953) |

[a]Some metabolites like the sterigmatocystin and aflatoxin precursors versicolorins, and all analogues of aspegillic acid and flufuran are not listed here.

Aflatoxins have never been found in true *A. oryzae* isolates; however, cyclopiazonic acid and β-nitropropionic acid are on the other hand toxins that are also found in *A. oryzae* (Barbesgaard et al., 1992; Blumenthal, 2004). Recently the list of *A. oryzae* (potential toxic) metabolites as expanded with the tremogenic mycotoxin 13-desoxypaxilline, an aflatrem precursor, and the lesser investigated parasiticolide A as well as aflavinines (Rank et al., 2010 in prep). With these recent discoveries, *A. oryzae* is also moving closer to *A. flavus* at the metabolomic level.
As noted by (Cleveland et al., 2009), there is an untapped potential for new chemistry and perhaps novel pharmaceuticals in *A. flavus*, despite the negative metabolic heritage.
One important point for searching for novel metabolites in *A. flavus*/*A. oryzae* species is to approach de-replication with all group *Flavi* metabolites as possibilities, as more metabolites apparently are shared amongst this group, than have previously been appreciated.

From the chemical overview of Table 1 (see also Figure 1) for group *Flavi* it is clear that the published knowledge about the subtle difference between these species is otherwise limited. Many of these species are also relatively newly assigned species and the collected data are therefore limited and the
definitions of the individual species may also be incomplete. A larger collection of these unique fungi will undoubtedly be important for the holistic perception of the chemistry in *Aspergillus* section *Flavi*.

## Results and Discussion

### Chemistry

As presented above, *Aspegillus* section *Flavi* is chemically a relatively homogenous conglomerate of fungi, having many metabolites in common, but with delicate variations they also represent a large chemodiversity. The *A. flavus*/*A. oryzae*-collection alone spans a very large diversity; however, the lack of many distinct differences makes chemotaxonomy less strait forward.

Our chemical analysis of the groups *Flavi* is based on a multitude of methods and instruments. Here we have used two orthogonal methods to assess the chemodiversity and evaluated their benefits and shortcomings as general methods for species identification within this section. Data for both types of analysis were microscale extracts based on a variety of plugs containing both new and old segments of the colonies (Smedsgaard, 1997a).

The first analysis was based on an unsupervised, multivariate clustering of direct injection mass spectrometry (DIMS) data. DIMS data have previously been shown to enable a clustering of different filamentous fungal species using intricate multivariate data analysis (Smedsgaard and Frisvad, 1996; 1997; 1997b; 2004; Hansen and Smedsgaard, 2007).

The second study focused only on *A. flavus*/*A. oryzae* isolates sampled with HPLC-UV/Vis DAD data and clustered with a supervised, binary correspondence analysis, based on a selected set of metabolites.

These methods are complementary to some extent, since the detection techniques are different and the latter approach uses chromatographic separation to resolve the selected compounds. The sclerotium identity of the isolates was an important factor in this study and we therefore analyzed the chemical profiles of a selected set of L and S-type sclerotia, to check for significant chemical differences between the two types. Novel, important metabolites identified in the clustering was targeted for isolation in structural elucidation, with common NMR and X-ray techniques.

### Selection of isolates and generation of data

In agreement with the OSMAC theory we probed the selected isolates of the different species on several different media (YES, YESBEE, DRYES, CYA, CYAS, CY20, CY40, DUL, GAK, GMMS, MEA, OAT, PDA, TGY, WATM (see Methods and Materials for explanation)) for selection of the best basis of optimal chemical

diversity. YES and CYA agar was chosen for the chemical analysis. YES agar generally promotes the highest aflatoxin production for these fungi, whereas CYA agar gives a more balanced chemical profile.

The morphology of the fungi on these two media reflects the chemistry, so that YES agar provides a relative uniform expression of color of the conidia and reverse, while the growth on CYA agar display more subtle differences in color variations. AFPA agar was chosen for the visual detection of

aspergillic acid, as it complexes with the ferri ions, resulting in a very orange reverse, for a positive result. Finally WATM media was selected to check the isolates ability to produce sclerotia and note the relative size of these.

We selected a broad collection of isolates, to our knowledge representing most of the diversity with *Aspergillus* section *Flavi*. In the separation of S-types, such as *A. minisclerotigenes* from *A. flavus*, the size of sclerotia is an obvious visual feature that allows for a quick separation of the two species. The small sclerotia-isolates used in this study were easy to distinguish from other isolates due to their extreme sclerotium production and very low conidiation. The distribution of the sclerotia was also very uniform for *A. minisclerotigenes* compared to *A. flavus*, where the sclerotia had a tendency to cluster 1-2 cm from the inoculums and exodate droplets where often observed and it occasionally covered clusters of sclerotia completely. The often difficult visual separation of *A. flavus* and *A. oryzae* led to some isolates being tentatively identified until chemical confirmation could be established.

The distribution of species selected for the DIMS and correspondence analysis is presented in Table 3.

**Table 3**. Statistics of the 381 isolates analyzed for DIMS cluster analysis and in parenthesis the strains used for the triplicate, and the 194 isolates of *A. flavus* and *A. oryzae* used in the correspondence analysis. Full list available in Appendix A.

| Species | DIMS (triplicate) | Correspondence analysis |
|---|---|---|
| *A. arachidicola* | 10 (2) | |
| *A. bombycis* | 3 | |
| *A. caelatus* | 2 | |
| *A. columnaris*[a] | 4 (2) | |
| *A. flavus* | 283 (20) | 151 |
| *A. kambarensis*[a] | 2 | |
| *A. minisclerotigenes* | 21 (3) | |
| *A. nomius* | 7 | |
| *A. oryzae* | 35 (4) | 43 |
| *A. parasiticus* | 1 (1) | |
| *A. parvisclerotigenus* | 3 | |
| *A. pseudotamarii* | 3 | |
| *A. sojae* | 1 | |
| *A. tamari* | 4 | |
| *A. terricola* | 2 (2) | |
| *A. toxicarius*[b] | 1 (1) | |

In addition to the real triplicates, 10 duplicates were added; 8 *A. flavus* and 2 *A. oryzae*.

[a] *A. columnaris* and *A. kambarensis* is regarded is genuine *A. flavus* strains, but officially not renamed yet.

[b] *A. toxicarius* is considered a synonym of *A. parasiticus*.

**DIMS analysis and model generation**

The first part for an in-depth analysis of the chemo diversity of *A. flavus* was to see if *A. flavus* and *A. oryzae* could be clustered separately from other section *Flavi* species such as *A. minisclerotigenes*, *A. caelatus*, *A. parasiticus* and other closely related species using the fast DIMS method. The second part was the extraction and analysis of which metabolites that gave rise to the clustering

Our general experience with *Aspergillus* chemistry and particularly that of *A. flavus* on the LC-ESI-MS system prompted for positive ionization mode as this is optimal for most metabolites from this group of fungi; particularly aflatoxins and CPA ionize well in positive mode. However, the important aspirochlorine is an example where negative mode is optimal why this compound is often not seen in crude extracts analyzed in positive mode. The inevitable loss of information by not re-running the dataset in negative ionization and not having a reverse phase column separation is counterweighted by the speed: one DIMS run was optimized to 3 min., whereas our standard HPLC method is 35 min. long including wash.

The DIMS method suffers from ion suppression for metabolites that ionizes strongly and are present in high amounts, as Figure 2 depicts.

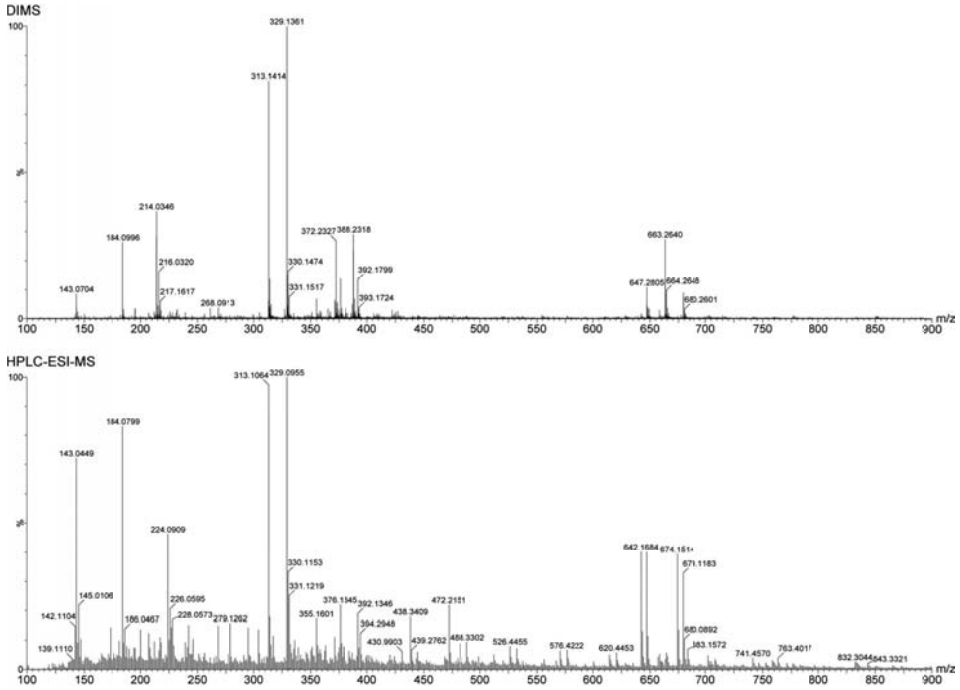


**Figure 2**. Top spectrum: DIMS, bottom: LC-ESI$^{+}$-MS. Both are simple TIC combined spectra without normalization or background subtraction. The ion suppression of the kojic acid and aflatoxins ($B_1$ and $G_1$ and dimers) is clearly evident, as many more metabolites are observable from the HPLC-MS data.

The data files were sampled in one continuous sequence with 20 randomized samples followed by an aflatoxin $B_1$ standard and two blank samples to normalize the dataset for instrument drift during the acquisition. A triplicate subset was randomly selected among the isolates for modeling and investigation of the DIMS method to differentiate these fungi. Two complete data sets were generated: one from YES cultivated isolates and one from CYA agar. For the following discussion only YES-data is analyzed, as the more uniform chemical expression of these data proved advantageous for the clustering in preliminary tests. Finally, the method described by (Hansen and Smedsgaard, 2007) was used, extracting the data from the raw datafiles, and lastly organising them into a grid that was suitable for a subsequent data analysis.

As the first step, the data were as filtered using analysis of variance (ANOVA) removing peaks individually with no significant information about any groups. Next, analysis of the variation present in the data,

through a Principal Component Analysis (PCA), revealed that the main variation in the data was due to the "weak" and "strong" isolates. The second and third modes of variation (principal components 2 and 3) on the other hand showed a good separation of the samples (see Figure 3) which can be emphasized further when overlaid with the physical non-correlated AFPA reactions and sclerotia size (see Figure 4A and B).

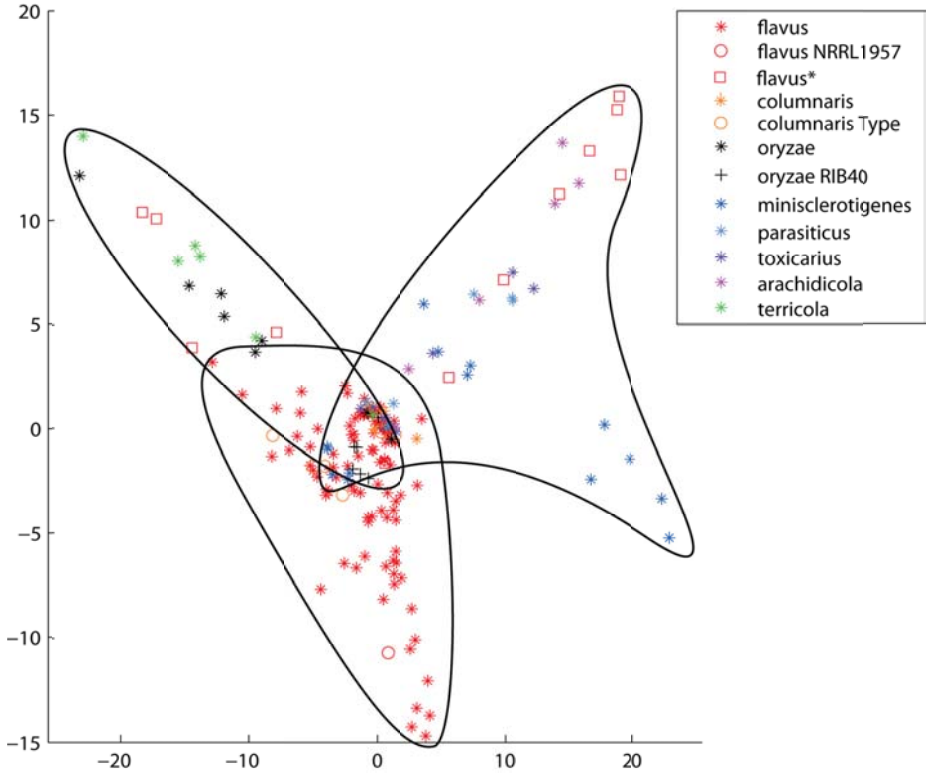


**Figure 3**. Filtered, unsupervised DIMS analysis of the triplicate subset. The *A. flavus* labeled with * were isolates with questionable history and identification was thus based solely on visual inspection.

The tendencies from the filtered, unbiased clustering from the data model, based on the triplicate subset, shows three major trends: one group correlating to the aflatoxin G-producers, one *A. flavus* group and an *A. oryzae*/*A. terricola* group. This result is in agreement with the general perception of these fungi as presented earlier. The relative high homology in chemical profiles of the *Flavi* members and the set of "weak" isolates does cause a portion of the samples to group in the middle of the plot. The tentatively identified *A. flavus* isolates in the *A. oryzae* and aflatoxin-G group respectively, can convincingly be regarded as miss-indentified *A. oryzae* isolates with an *A. flavus*

appearance and an aflatoxin-G producer, likely *A. nomius* which can be morphologically mistaken for *A. flavus* if it is less floccose. LC-MS analysis confirmed this.

*A. oryzae* tends to cluster to the upper left, although the RIB40 isolate is placed more centrally. The grouping with *A. terricola* is also expected from the "non-toxigenic" profile listed in Table 1. Also *A. flavus* and *A. columnaris* (sub group of *A. flavus*, which can be regarded as a normal *A. flavus*) are showing some clustering towards the bottom. *A. minisclerotigenes*, *A. nomius*, *A. arachidicola*, *A. toxicarius* and *A. parasiticus* all seem to correlate at the upper right of Figure 4A. *A. toxicarius* is likely going to be transferred to *A. parasiticus*, which correlates with these observations. When overlaying the plot with the observed reaction on AFPA media, as an indication for the production of aspergillic acid, there is a tendency for non-production towards the *A. oryzae* grouping and a varying degree of APFA reaction in the other two groups (Figure 4A). If the size of sclerotium produced on any media is overlaid these results, there is a good correlation between the sclerotia size and clusters, as we expected (Figure 4B). However, this result reveals that there is a fundamental chemical difference between *A. flavus* and the S-types, which correlates to sclerotia size L and S. The samples used were all 7 day old cultures, but as some isolates display a slower and less abundant sclerotium production, some of the 0-labeled isolates might still be able to produce sclerotia given enough time or other growth conditions.

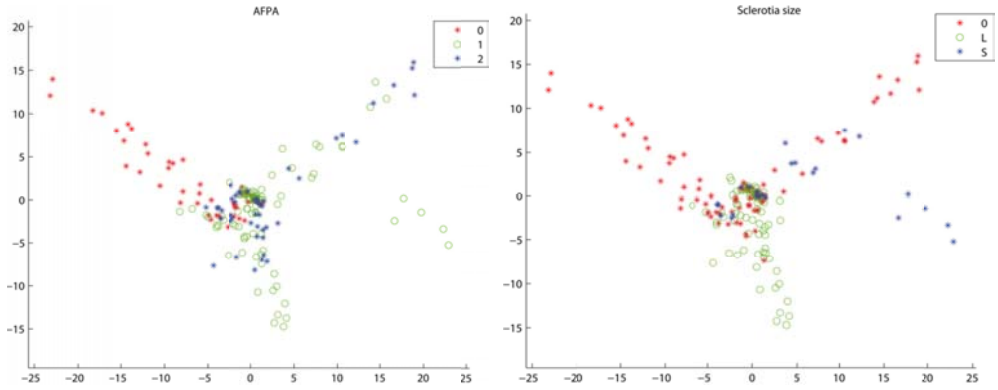


**Figure 4**. A and B. Plot A presents the filtered, unsupervised clustering based on DIMS data overlaid with observations for A) reaction on AFPA media in the range none to very little reaction (0), medium (1) and strong (2). B) size of sclerotia observed on any media after 7 days cultivation: 0 for observed sclerotia, L for large and S for small.

To investigate the influence of *presumed* important and known metabolites a supervised analysis of the data basically using a general list of *A. flavus* metabolite was performed. We basically applied the list of known metabolites from literature presented in Table 2, and included aflatoxin G's, that are

important metabolites for the S-type isolates. The results are presented in Figure 5. The three group tendencies are present, as in the filtered, unsupervised clustering: *A. flavus*, aflatoxin G-producers and *A. oryzae* are now much more separated and again the results can be validated with the overlays of AFPA reaction and sclerotia sizes. For some for the *A. minisclerotigenes* samples a better separation was possible, though some of the *A. minisclerotigenes* isolates suffer from being "weak". It was not possible at this point to normalize data and compensate for the apparent concentration difference of the samples and emphasize the chemical variations.

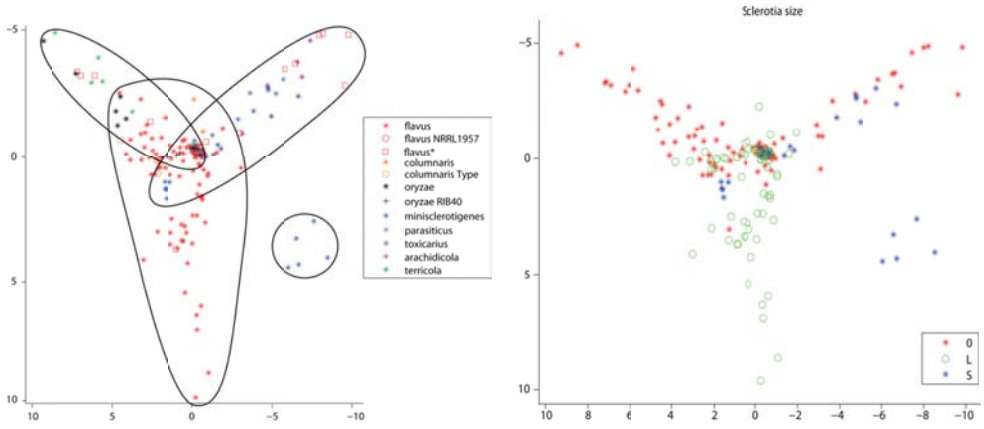


**Figure 5**. A and B. Development of the model based on the triplicate subset. Plot A presents the broad supervised clustering of the DIMS data. B shows the same plot overlaid with observations for size of sclerotia observed on any media after 7 days cultivation: 0 for observed sclerotia, L for large and S for small.

The correlation of the filtered, but unsupervised model with the literature-based supervised approach proves that the list contains the most important metabolites for the DIMS clustering and that it is truly significant metabolites for the separation of the species. It is intriguing that the chemodiversity of *A. flavus* is so large compared to the several other species in the aflatoxin-G group. This is again consistent with the genetic observation of many different VCG's and types of mutants for this species alone (Barros et al., 2005; 2006; Ehrlich et al., 2007; Horn, 2007).

To test the model and potentially improve the clustering we first analyzed the triplicate dataset with LC-MS manually and particularly targeted the most abundant metabolites and those which proved dominant in the DIMS data files. Together with the triplicate analysis, a selective extraction of a set of washed L- and S-type sclerotia was carried out using different extraction procedures and analyzed with HPLC-UV-MS. No significant differences was observable between the two types of sclerotia, besides the inherent separation of aflatoxins, with only aflatoxin B's in L-sclerotia and massive

amounts of B and G in the S-type, thus the sclerotia chemical profile could not be used to differentiate L- and S type *Flavi*. For the differentiation of sclerotia or non-sclerotia producers, the typical apolar metabolites of sclerotia, like alfavinines, kotanins, and aflatrem could be beneficial. The shear amount of aflatoxins and the selective production of aflatoxin G's (save *A. parasiticus*; L-sclerotia) were also an important part of the DIMS clustering. The selected metabolites are listed in Table 4. These metabolites could to some degree be more important for the DIMS analysis due to the ionsuppression discussed earlier, as the most dominant metabolites will diminish the less abundant and poorly ionizable metabolites.

**Table 4**. Important metabolites targeted for a highly supervised analysis of the DIMS data.

| |
|---|
| Aflatoxin $B_1$, $B_2$, $G_1$ and $G_2$ |
| Sterigmatocystin, O-methylsterigmatocystin |
| Cyclopiazonic acid, speradine A |
| Kotanin, demethylkotanin |
| 10,23-Dihydro-24,25-dehydroaflavinine, 20,25-dihydroxyaflavinine, aflavinine, nominine |
| Aflatrem |
| Flavimine |
| Kojic acid, kojic acid dimer |
| Stachydrine (proline betaine) |
| Aspergillic acid |

The results of the highly supervised model showed (figure not shown) a less good separation than presented with the other methods. Clearly the selected metabolites are the dominant metabolites and are important for the clustering and chemical separation of the different species of *Aspergillus* section *Flavi*, but the subtle peaks and data variation is also vital for a proper clustering.

**Peak listing and comparison to metabolite lists**

Although using a high-resolution mass spectrometer (HR-MS), it can be difficult to extract exact masses from the DIMS data, as they must be automatically detected during the initial steps of converting the raw data, before the data analysis step. In this study, the centroid (intensity and mass) were estimated and used in the data analysis. With the statistical significant clustering of the triplicate dataset, combined

with our knowledge of unique metabolites for each group, such as aflatoxin G, it is possible to review the extracted masses and give a tentatively identification of the peaks (Table 5).

The most probable and significant compounds compared to the prerequisite knowledge listed in Table 2, are listed in Table 5. Differentiation in adducts is possible, such that the $[2M+H]^+$ ion might be significant for the clustering and therefore listed, while the $[M+H]^+$ or $[M+Na]^+$ is not, and there

for absent in this data extraction. This is partly due to the mechanisms in the ionsource where $[M+H]^+$ adducts are not always predominant.

The list of metabolites generated for group 1 (*A. oryzae*) was primarily low molecular weight peaks, which corresponds with our perception of this species as domesticated and generally less pronounced secondary metabolite producer. Maltoryzine and oryzaecidin are primarily known from *A. oryzae* and therefore plausible differentiating metabolites.

Group 2 were the potent aflatoxin G producers and especially compounds in the aflatoxin pathway appeared important for the clustering of this group, as aflatoxin $B_{1,2}$, $G_{1,2}$, and two sterigmatocystin analogues could be tentatively identified from the generated list of significant metabolites. Aspergillic acid and CPA was likely also dominant peaks. All of these metabolites can be indirectly validated with the observed strong AFPA reactions (aspergillic acid) and partly strong production of (S) sclerotia, as aflatoxins and CPA are strongly correlated to this structure.

Group 3 was the *A. flavus* isolates were dominated by sterigmatocystin analogues, ditryptophenaline, speradine A, aflavinine and asperantin, which are all known from the species and as for group 2 also correlates with the (L) sclerotia production. The very broad chemodiversity of this species is likely to be overshadowed be the dominant peaks for group 2. The occurrence of the CPA analogue speradine A in the *A. flavus*-cluster indicates a higher significance of this metabolite for the differentiation of this species, compared to the CPA-production for group 2.

**Table 5**. Important, clustering peaks from DIMS analysis and tentative identification.

| *Group 1 – A. oryzae/A. terricola* | | *Group 2* – Aflatoxin G-producers | | *Group 3 – A. flavus* | |
|---|---|---|---|---|---|
| Mass | Metabolite | Mass | Metabolite | Mass | Metabolite |
| 232.1107 $[M+Na]^+$ | Maltoryzine | 313.2393 $[M+H]^+$<br>330.2721 $[M+NH_4]^+$ | Aflatoxin $B_1$<br>Aflatoxin $B_1$ | 324.3280 $[M+NH_4]^+$ | 6-O-Methylasperentin |
| 156.3036$[M+H]^+$<br>173.1672 $[M+NH_4]^+$ | (2,3-Dihydro)-3-hydroxyanthranilic acid | 332.2712 $[M+NH_4]^+$ | Aflatoxin $B_2$ | 385.3010 $[M+H]^+$<br>426.3241 $[M+MeCN+H]^+$ | 5,6-Dimethoxy-sterigmatocystin |
| 166.1593 $[M+Na]^+$ | Kojic acid | 329.2393 $[M+H]^+$ | Aflatoxin $G_1$ | 405.3654 $[M-H_2O+Na]^+$ | Aflavinine |
| 204.1825 $[M+H]^+$<br>221.2107 $[M+NH_4]^+$ | Oryzecidin<br>Oryzecidin | 331.2704 $[M+H]^+$ | Aflatoxin $G_2$<br>Aflatoxin $G_2$ | 716.6612 $[M+Na]^+$ | Ditryptophenaline |
| | | 644.6187 $[2M+Na]^+$ | Demethyl-sterigmatocystin | 367.2860 $[2M+H]^+$ | Speradine A |
| | | 355.3072 $[M+H]^+$<br>709.4245 $[2M+H]^+$ | 5-Methoxy-sterigmatocystin | | |
| | | 314.2730 $[M+H]^+$ | Cyclo(D-N-methyl-Leu-L-Trp) | | |
| | | 472.2929 $[2M+Na]^+$ | Aspergillic acid | | |
| | | 393.3465 $[2M+H]^+$ | Dipropyl neoaspergillic acid | | |
| | | 422.3098 $[M+H]^+$ | 20-Hydroxyaflavinine | | |
| | | 673.2990 $[2M+H]^+$ | Cyclopiazonic acid | | |

The results of the DIMS analysis is to some extent ambiguous, in the sense that there is no strict boundary between the three clusters as we expected: especially the separation of *A. oryzae* and the aflatoxin G-producers would ideally have been clearer.

A preliminary conclusion from the DIMS model set is that it is able to describe the chemical tendencies within section *Flavi* and that a fractional clustering is possible. The method limitation is, however the grouping of chemically closely related fungi when the subtle differences are partially subsided because of the crude injection into the mass spectrometer. The dominant variation in the dataset is the relative concentration produced by each isolate (consistently in the triplicates) and one way to improve the model would be to understand the sources of this (biological) variability and use it to correct for it, and obtain an improved clustering. Use of less complex media formulation than YES agar, with a restriction of possible epigenetic modifiers from media components (yeast extract), might also be important for improvement of this method.

**Selective correspondence analysis of *A. flavus* and *A. oryzae***

While DIMS is fast and (partially) informative in the global section *Flavi* analysis, it suffers from lack of chromatographically resolving power and the technique breaks down to some extend when differentiating the important species *A. flavus* from *A. oryzae*. This is partially a logical consequence of the genetic and (expected metabolic) coherence and partially due to ion suppression and no chromatographic separation, and there are therefore delicate, valuable differences which can be made out using HPLC-UV-(MS).

We analyzed dataset of pure *A. flavus* and *A. oryzae* isolates selected partially on the chemical separation from the DIMS analysis using a supervised approach based on 29 selected markers (single metabolites, compound classes and sclerotia production, see appendix B), based on unique UV-chromophores and retention indexes. The data were generated from HPLC-DAD analysis of microscale extractions of these isolates and analyzed using correspondence analysis (Hill, 1974; Krzanowski, 1993) to extract the correlation of the selected metabolites. The metabolites were primarily well known compounds from Table 2, but characteristic, tentatively unknown metabolites were also selected and used in the analysis. Sclerotia production was in contrast to the DIMS analysis, were included in the correspondence analysis to allow for a weighing of the *potential* of sclerotia related metabolites. Data was perceived as binary and collected from several media (YES, CYA, OAT, WATM).

The grouping of isolates in this analysis confirms the complex picture presented by the DIMS data: namely a fluid transition from *A. flavus* to *A. oryzae* with a partial segregation of *A. oryzae* isolates from the majority of *A. flavus* isolates. The ex type of *A. flavus* (NRRL 1957) and the genome sequenced isolate (NRRL 3357) are relatively close in the bi-plot (Figure 6), emphasizing the many similarities rather than the emblematic aflatoxin divergence. The genome sequenced *A. oryzae* (RIB40) is placed close to the “border” against *A. flavus* and this is in agreement with our general perception of this specific isolate as chemical potent (Rank et al., in prep., chapter 6). The identity of this important strain is especially important, and contrary to the definition “wild type” *A. oryzae* as by Machida (Machida et al., 2008), the chemical perception of this isolate might rather be that is a non-aflatoxigenic *A. flavus*, as it is not from koji starters, but isolated in nature from cereal.

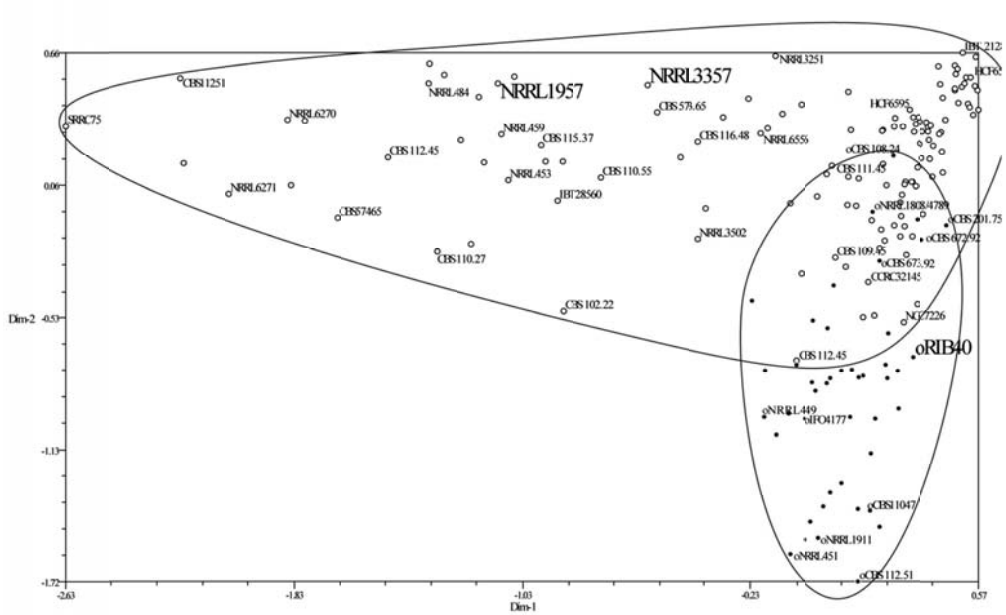


**Figure 6**. Correspondence analysis of *A. flavus* (circles) and *A. oryzae* (dots) isolates based on 28 unique metabolites and sclerotia production. The fluent transition from *A. flavus* isolates to *A. oryzae* is evident and emphasizes how they are difficult to separate chemically, when comparing to weak *A. flavus* isolates.

The examination of grouping in amongst the selected metabolites, reveals more to the story (Figure 7): three major groups can be identified in the dataset, representing core *A. flavus* metabolites, important *A. oryzae* metabolites, and a group of special *A. oryzae* metabolites. The core *A. flavus* metabolites are kojic acid, aspergillic acid, aflatoxin $B_1$ (and versicolorins for strong aflatoxin B producers), aflatrem, aflavinines, and three unidentified metabolites: FNI, EPIF and "flavimine". Kojic acid is expectedly placed in the origin as it is shared among most of the examined isolates; generally fewer *A. oryzae* strains produce kojic acid. Kojic acid is a growing concern in food production as addressed by (Tanaka et al., 2002; Blumenthal, 2004). Aspirochlorine and asperfuran are placed opposite to aflatoxin $B_1$, correlating more to the *A. oryzae* isolates (Figure 7). The group of important *A. oryzae* metabolites constitutes aspirochlorine, asperfuran, parasiticolide A and B, citroisocoumarin, cyclopiamine (tentatively identified) and four unidentified metabolites: PAL, NO2, NB and an indol-containing metabolite.

With the projection of the metabolites onto the distribution of fungal isolates it is clear that they are divided into an upper *A. flavus* segment and a lower *A. oryzae* group. The *A. flavus* segment is primarily concentrated around the core metabolites, but a portion – including the two important isolates mentioned before (NRRL 1957 and 3357) – lies more scattered to the left. These isolates

synthesize the special *A. flavus* metabolites along with most core components, but very few isolates produce these unique metabolites without the majority of the core metabolites.

The isolate SRRC75 produces primarily the group species metabolites (FUT, PRS, VERNO, FLIT and GYLE), and is placed at the very left side of the plot. There has been some dispute about the identity of this isolate: it was original an *A. parasiticus* type culture that was later proven contaminated with *A. flavus* (Kozakiewicz, 1982). In this analysis it differentiates strongly, as it produces primarily the "special" group of *A. flavus* metabolites and almost none of the core metabolites.

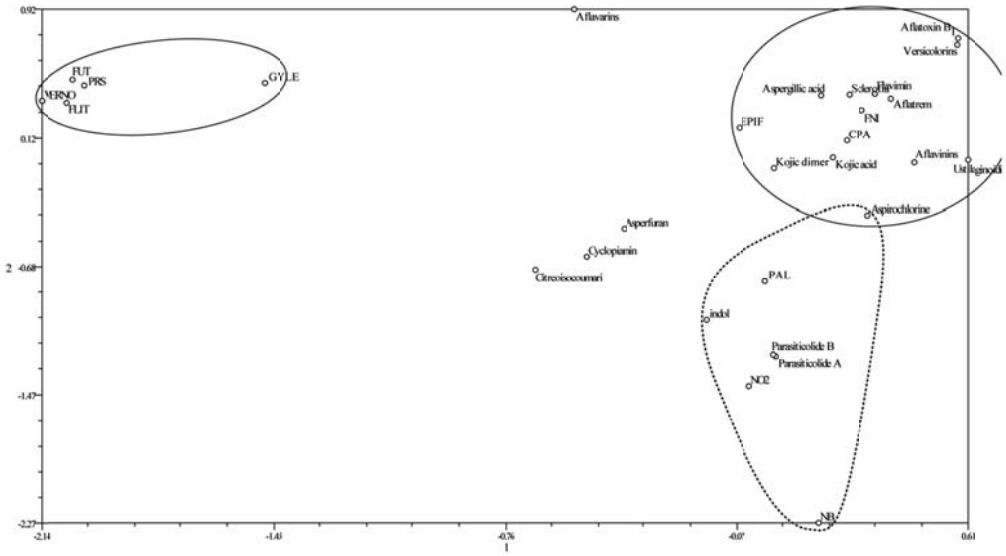


**Figure 7**. Correspondence analysis of the selected 28 metabolites (+sclerotia) and their coherence. Three major groupings are marked: The core *A. flavus* metabolites like aflatoxin $B_1$, aflatrem and aspergillic acid; a unique subset of metabolites (VERNO, FLIT, GYLE, FUT, PRS), important for some *A. flavus* isolates; and a looser grouping of *A. oryzae* separating metabolites (dashed).

The isolate NRRL 3251 has been reported as both a S- (Egel et al., 1994) and a L-sclerotia producer and might be borderline in terms of sclerotium diameter. We have only observed large sclerotia and the general chemical profile also implies that it is merely an average *A. flavus* isolate, consistent with the central placement in the clustering.

The three known isolates of *A. flavus* var. *effusus* (CBS 574.65, CBS 110.27 and CBS 102.22) are all placed in the lower middle part of the *A. flavus* group.

The two cultures labeled *A. chungii* (CBS 115.37 and CBS 118.63) are here grouped close to the two important *A. flavus* isolates NRRL 1957 and NRRL 3357. We believe that they are simply *A. flavus* strains, though they appear under *A. parasiticus* in the CBS strain catalogue.

The *A. oryzae* isolates are all valid industrial isolates, which in essence is the definition of the species. From Figure 6 is apparent how the two species intertwine and that there is no clear separation. Some metabolites like parasiticolides, NB and NO2 seems to be more important for the clustering of the *A. oryzae* isolates, whereas asperfuran and aspirochloride are less significant, as they are also present in some *A. flavus* isolates. The inconsistent kojic acid production is also influencing the move away from *A. flavus* core metabolites.
Those isolates of *A. oryzae* that are closest to the *A. flavus* core: CBS 108.24, NRRL 1808, NRRL 4789, CBS 672.92 and CBS 673.92 are all genuine koji starter cultures. The strain NRRL 449, was included in the study by (Geiser et al., 2000), where it consistently grouped (IB) together with other *A. oryzae* and some *A. flavus* isolates (L-type) in the phylogenetic study based on selected aflatoxin genes.

**Chemical markers**
The DIMS analysis of *A. flavus/A. oryzae* against more distant species, such as *A. minisclerotigenes* and *A. parasiticus*, has revealed those chemical markers that are important for the clustering. Especially aflatoxin G is a prominent metabolite in this study, since it is produced in high amounts by the species capable hereof and it ionizes well in the ion source of the mass spectrometer.
The separation of *A. flavus sensu stricto* from *A. oryzae* is more difficult and the chemical differences are also difficult to pinpoint; *A. flavus* is often not producing aflatoxin or sterigmatocystin, and as *A. oryzae* is occasionally able to produce CPA, and these important toxin are therefore not a solution to segregate the species.
The DIMS and correspondence analysis of many isolates of both species has resulted in the discovery of metabolic markers for *A. flavus*. These apparently novel metabolites were further verified with LC-MS analysis of the triplicate dataset. The CPA analogue speradine A has not previously been reported from *A. flavus*, but only from a marine-derived *A. tamarii* strain (Tsuda et al., 2003). It appears to be an important metabolite both for the identification of *A. flavus* as well as part of the general chemical potential. Many isolates are able to produce this compound in reasonable amounts. The compound was isolated from a YES fermentation of the ex type culture (NRRL 1957=IBT 3610) and the structure was confirmed with NMR. Several analogues of CPA was co-isolated as minor components, but proved unstable during acquisition of NMR data. Speradine A has mainly been observed in isolates that was producing CPA and it is possible that the metabolite is a bi-product of this biosynthesis, although two isolates (IBT10963 and IBT21070) produced roughly equal amounts of both, indicating a more distinct function for speradine A, than just a bi-product. The ex type culture was the only examined isolate that produced speradine A without traceable (LC-MS) CPA production.

Asparasone A, an anthraquinone pigment, was de-replicated with LC-(ESI$^-$)-MS and found in several isolates of the subset, but previously it has only been known from *A. parasiticus* (Sobolev et al., 1997). The metabolite was hypothesized by Sobolev et al. to be linked to the aflatoxin biosynthesis, as the structure resembles the basic structure of norsolorinic acid, the first stable intermediate towards sterigmatocystin and aflatoxin. The side chain is however two carbons short, as seen in Figure 8. Interestingly, several non-aflatoxigenic isolates, including *A. flavus*$^T$ were found to produce asparasone A. As the metabolite is only observable in ESI$^-$ mode, it did not influence the clustering, but might prove important for chemotaxomical purposes.

**Figure 8**. Norsolorinic acid and the structurally related asparasone A.

The novel "flavimine" (in terms of HR-MS de-replication) has a uniquely identifiable UV-chromophore similar to the diketopiperazine aurantiamine (Larsen et al., 1992) (see Figure 9), is an even more unique metabolite to *A. flavus* and an impending chemical marker for *A. flavus* alone, as we have not seen this metabolite in other species within section *Flavi*. The compound could be a product of one of the two putative nonribosomal peptide synthase (NRPS) found in the *A. flavus* genome, found to be unique compared to *A. oryzae* (Rokas et al., 2007).

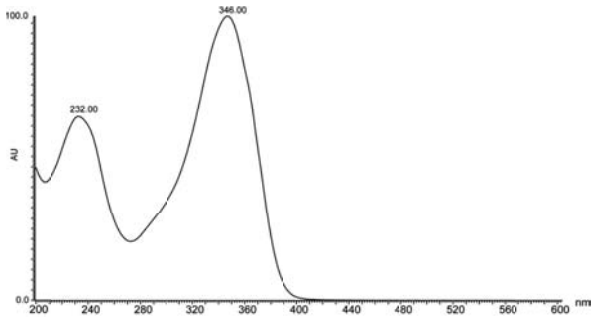


**Figure 9**. UV chromophore of the novel *A. flavus* chemical marker "flavimine".

The less pronounced secondary metabolite profiles of some *A. oryzae* strains were important for the clustering of these isolates. This emphasizes the problem of ionsuppression, as some of these lower molecular constituents might be present in a number of *A. flavus* isolates, but are suppressed and

thus insignificant for the variation in data. Many of the low molecular or otherwise important metabolites of *A. oryzae* are better ionized in negative mode, thus not influencing the DIMS analysis. The de-replication of NB and NO2 did not produce hits in database searches against known, potential compounds, but the identity of these markers are undoubtedly important for the understanding of *A. oryzae.*

The scattering of isolates in the correspondence analysis is influenced by the metabolites chosen and relative biosynthetic correlation: if all metabolites were structure elucidated, the analysis could be changed to represent active biosynthetic routes, rather than single metabolite identities. The similarity of the different isolates would presumably be much more correlated with this approach, but the subtle differences in the metabolite profiles and the underlying regulation would be lost. The presence of the aflatrem-precursor 13-desoxypaxilline in *A. oryzae* (RIB40) and no traces of aflatrem itself is an example of this point (Rank et al., in prep, chapter 6).
The presented chemical diversity has one other important implication for the *Aspergillus* community, namely the foundation for a phenotypic selection of the next isolates to be genome sequenced: *A. flavus* SRRC75, *A. oryzae* CBS 108.24 and CBS 112.51 are all extreme isolates in terms of chemical profiles compared to the present genome isolates and a better understanding of the *A. flavus*/*A. oryzae* genome would presumable gain much added insight on expression, among many other issues. The general approach to use the chemodiversity, hence the phenotype to select isolates also within other species is definitely an important criteria, especially for the understanding and expression of a given species full chemical potential.

**Domestication**

The close correlation between *A. flavus* and *A. oryzae* at the genetic level, but as shown in this study also for phenotypic expression in terms of chemistry, have resulted in the general perception of *A. oryzae* as a domesticated variant of *A. flavus* (the wild type). The natural environments for *A. flavus* are (ground)nuts, spices, crops and occasionally dried fruits and the is species is also a human pathogen. In contrast *A. oryzae* is per definition only sampled from fermentations, although reports have been published on soil and plant samples, but these isolates where most likely *A. flavus* variants resembling *A. oryzae*, having slightly more floccose mycelia and a more yellow color tone conidia coupled with non-aflatoxin production. But how are *A. oryzae*-like *A. flavus* isolates to be distinguished from *A. oryzae*? The origin of the *A. oryzae* isolates used in industry have once been derived from wild type *A. flavus* isolates and have through long use in very controlled environments been *directly* domesticated to be used in specific fermentations. The secondary metabolites have generally been down regulated or completely silenced and the production to proteolytic enzymes has been up regulated ex for the initiation of starch break down. Genes coding for those metabolites that appears unique to *A. oryzae* are without doubt present in the genome of *A. flavus*, but potentially not expressed under normal conditions to a detectable level due to a different regulation

focused on the environmental challenges presented to *A. flavus* in nature; insects and other microorganisms. Production of the soil surviving sclerotia has almost been eradicated from *A. oryzae* together with the sclerotia associated metabolites, and is only found in two of the isolates we have analyzed (one being RIB40). Our explorative study of the chemical content of sclerotia indicates increased levels of aflatoxin and CPA alongside the normal sclerotia metabolites like aflavinines, kotanins and coumarins, aflatrem. The ecological function of aflatoxin and CPA has not been clearly established, but some indications points towards anti-insect mechanisms, which follows the line for the aflavinines and correlated metabolites. The atoxic appearance of *A. oryzae* could thus be explained with change of environmental and evolutionary pressure, promoting enzyme production over secondary metabolites. This is indirectly supported by the *A. oryzae* production of the potent phytotoxin aspergillimarasmine, also known from *Fusarium*, which has yet to be reported from other group *Flavi* members. The original article by Robert et al. mentions the isolate as an *A. flavus* var. *oryzae* with no references to strain number, so it is not possible to check the identity of the strain (Robert et al., 1962). The reason for CPA production in *A. oryzae* when there is no evolutionary pressure for its defense properties is likely because it functions as a trace metal scavenger (Gallagher et al., 1978; Laursen et al., 2009) for its own primary metabolism, together with aspergillic acid (Perry et al., 1984; Nurchi et al., 2009), aspergillimarasmine A (Barbier et al., 1963; 1987), and kojic acid (Bentley, 2006), which have all been found to complex with ferri ions, perhaps this feature applies β-nitropropionic acid as well.

Many sampled isolates of *A. flavus sensu stricto* have a high degree of inconsistency in aflatoxin-production and they are mainly found in crop fields that could be regarded as a semi-controlled ecological niche, where insects might be less abundant, thus lowering the pressure for aflatoxin production. In contrast to this variable production, *A. minisclerotigenes* are morphological and chemical highly homogeneous with extreme production of sclerotia and aflatoxin. These isolates, together with *A. parasiticus* have mainly been isolated from peanuts and insects and less from soil, but not from crops.
Chemically one could perceive *A. flavus* as an *indirectly* domesticated species of *A. minisclerotigenes* en route for an *A. oryzae*-like phenotype.
To understand the domestication mechanism from a chemical perspective, the tendencies in the metabolite profiles of *A. minisclerotigenes*, *A. flavus* and *A. oryzae* imply some notable features: the ability to synthesize aflatoxin G production is clearly lost in the speciation from *A. minisclerotigenes* to *A. flavus*, but apparently the overall regulation of this gene cluster is also affected and turned more labile. The domestication of *A. flavus* to *A. oryzae* results in an aflatoxin-defective variant, but accompanying this chance is also an up regulation of enzyme production and a shift in the secondary metabolome.

The series of yet unidentified *A. flavus* metabolites appear more limited to this species, as we have not seen these in *A. oryzae* isolates. With the direction of the domestication, it is more likely that there will be unique *A. flavus* metabolites than is the case for *A. oryzae*.

## Conclusion

*Aspergillus* section *Flavi* has an interesting and large variation. There is clearly a trend in loss of apparent toxicity from the uniform *wild type*, earthbound, small sclerotial types such as *A. minisclerotigenes*, over the (average) weak aflatoxin producer *A. flavus* with large sclerotia that thrives in crop fields and partially on plants themselves, to the GRAS organism *A. oryzae* used in biotech industries.

The holistic chemical survey of *A. flavus* presented here proves how the phenotype could be an important commencement for choosing the next strains for genome sequencing – what metabolite makes the outliers unique? Certain metabolites appear to be important for the clustering of these types of species, when using the DIMS methodology together with a correspondence analysis of HPLC-UV/Vis DAD data. They can be correlated to existent knowledge about the delicate differences between species within section *Flavi* and within the *A. flavus*/*A. oryzae* group. Speradine A and flavimine are new metabolites to *A. flavus* and holds a potential to be important biomarkers for the species together with a set of core metabolites. A unique group of metabolites exists for *A. flavus* and are expressed under normal circumstances for some isolates, that have previously been identified as *A. flavus* related species, but that we believe is part of the biodiversity within the species. Flavimine is potentially a diketopiperazine compound and thus could be one of the putatively annotated NRPS in the *A. flavus* genome.

*A. oryzae* is heavily overlapping *A. flavus* in both cluster analysis and this chemical coherence emphasizes the established genetic homology. Distinct differences make them partly separable, such as the unique and potentially novel metabolites NB and NO2. It is doubtful whether they will prove unique to *A. oryzae*, but under normal growth conditions they do not appear in any *A. flavus* isolates.

**Future perspectives**

Before the DIMS model can be applied to the full set of isolates listed, further validation must be performed, as well as a better understanding for weak and strong isolates and their influence on the clustering must be obtained. With a better filtering for important and u nique peaks for each of the groups, it might be possible to produce a better clustering, which will be important for the extension to a larger dataset.

New UPLC technology promises much improvement for the standard LC-MS method with acquisition 2-3 times faster, but the generated data files will still be much larger than DIMS files, as the resolution and scan frequency must be increased for the mass spectrometer as well. The improved MS instruments and autosamplers will also provide possibilities for refinement of the DIMS method and perhaps enable a better separation of very closely related species in the future.

Ultimately, a secondary metabolome study should be conducted in connection with transcriptomics and bioinformatic analysis to enable a better understanding of the link between the geno- and phenotypes of these important fungi.

## Materials and Methods

### Growth conditions

The validated isolates from the Culture Collection were inoculated as three point cultures on 9 cm Petri dishes with 17 mL substrate agar of CYA (Czapek Yeast Autolysate), YES (Yeast Extract Sucrose), WATM Wickerhams Antibiotic Test Medium) agar (Raper and Thom, 1949), YESBEE (YES+50g Bee pollen Type III, granulate, Sigma, P-8753, pr. 1L medium), DRYES (Dichloran rose Bengal chloramphenicol agar), AFPA (*Aspergillus flavus*, *A. parasiticus* agar), CYAS (CYA+50g NaCl pr. 1L medium), CY20 (CYA+200g sucrose pr. 1L), CY40 (400g sucrose pr. 1L medium), DUL (Dulaney's medium for Penicillin), GAK (Potato-carrot agar), GMMS (Glucose minimal media (GMM) +2% sorbitol), MEA (Mout extract agar), OAT (Oat meal agar), PDA (Potato-dextrose agar). For medium formulations see (Samson et al., 2004).
The cultures were grown for 7 days in dark at 25°Celsius in stacks of 8 in micro-perforated bags.

### Microscale extractions

Micro-scale extractions were prepared using a variation of the method described by Smedsgaard (Smedsgaard, 1997a): The cultured isolates were checked morphological and 5 plugs of 5 mm was collected in a 2 mL glas vial and stored at -20°C. There plugs were added 1 mL of a solvent mixture: 1:2:3 methanol:dichloromethane:ethylacetate with 0.5% formic acid and ultrasonicated for 45 min. and transferred to a clean glass vial. After evaporation overnight in a fume hood, 0.5 mL methanol was added and the samples ultrasonicated for 10 min. for re-dissolving the solids and then stored at -20°C until analysis.

### Visual inspection and data sampling of DIMS dataset

25 isolates where analyzed visually and sampled at a time. All samples were run in one sequence continuously over 5 days. For each 20 samples one aflatoxin $B_1$ standard followed by a blank was run to follow the drift of the instrument.

### DIMS method

LCT oaTOF mass spectrometer (MicroMass, Manchester, UK) with a Z-spray ESI source and a LockSpray probe fitted with an Agilent HP1100 auto sampler (Waldbronn, Germany). All solvents used were HPLC grade from Sigma-Aldrich (St. Louis, MO, USA) and water was filtered using Milli-Q (Millipore, Millford, USA) with a 0.22 µm PTFE filter. The method was adapted from the general procedures of Smedsgaard and Frisvad (Smedsgaard and Frisvad, 1996): 0.100 µL of the extract was inject directly onto the LCT with a flow of 0.300 µL 50:50 water:acetonitrile in the 3 min. The triplicate samples were run in beginning and end of the entire sequence. Masses were measured in range m/z 100-1000 Da. Spectra were collected at

a rate of 1 spectrum per second from m/z 150 to m/z 1000 in continuum mode. The Lock mass during scanning was a leucine-enkephalin solution (0.2 µg/ml in acetonitrile-water-formic acid; 50:50:0.1) and the $[M+H]^+$ was set to 556.2771 Da/e with a reference scan every third scan. (Nielsen and Smedsgaard, 2003; Frisvad and Samson, 2004)
Solvents 50% MeCN at a flowrate of 0.300 mL/min. Stop time 3.0 min. Injection Volume 3.0 µL, eject speed 3.0 µL/min. Nebuliser gas flow 17 L/hr, desolvation gas flow 420 L/hr. Capillary 3000 V, sample cone 25 V, RF lens 300 V, extraction cone 5.0 V, desolvation temperature 400 C, source temperature 120 C. Data was sampled with Waters Masslynx v4.0.

HPLC with diode array detection and high resolution mass spectrometric detection (HPLC-DAD-HRMS), was performed on an Agilent 1100 system with a Luna C18(2) column (Phenomenex, Torrance, CA) and equipped with a photo diode array detector (DAD), and coupled to a LCT orthogonal time-offlight MS (Waters-Micromass, Manchester, UK), with a Z-spray ESI source and a LockSpray probe (Nielsen et al., 2009). Furthermore all isolates were analyzed by HPLC-DAD using the method of (Frisvad and Thrane, 1987; 1993) and modified by (Smedsgaard, 1997a). Data was sampled with Waters Masslynx v4.1.

**Isolation and physical data of speradine A**

The compound was isolated from a 14 day old fermentation of 200 plates *A. flavus* IBT3610 (NRRL 1957) on YES agar (25°C, dark). The agar plates were homogenized using a Stomasher and extracted with EtOAc with 1% formic acid. The extract was filtered after 24 hours passive extraction and concentrated on a Büchi Rotavapor R-134 with a Büchi Waterbath B-480 rotavapor. The extract (19g) was resolved in MeOH and dried down on the rotavapor with a portion of C18 material. The C18-bound extract was run through a C18 50µm 150 x 40 mm (135 g) Phenomenex column in 10% step gradient of MeOH:$H_2O$ starting at 10%. The 80% fraction contained concentrated speradine A and the compound was isolated using a Waters C18 15µm 300 mm x 19 mm HPLC column on a Waters 600 system with Waters 996 photo array detector using a gradient of 40-67% MeCN over 30min. Solvents was added 50ppm TFA.
The mass was measured at $[M+H]^+$=367.16690, ($[M-H]^-$=365.14780), calcd. for $[C_{21}H_{22}N_2O_4+H]^+$ 367.16522. The NMR data were acquired in DMSO-$d_6$ at 499.87 MHz ($^1$H) and 125.71 MHz ($^{13}$C), respectively, on a Varian Unity Inova. Spectra were referenced according to solvent resonances at $\delta_H$ = 2.50 and $\delta_C$ = 39.43 ppm. $^1$H NMR δ 7.18 (1H, dd, *J*=7.6 and 7.6 Hz, H15), 6.88 (1H, d, *J*=7.6 Hz, H14), 6.81 (1H, d, *J*=7.6 Hz, H16), 4.51 (1H, d, *J*=7.7 Hz, H5), 3.62 (1H, d, *J*=5.4 Hz, H3), 3.09 (3H, s, *J*=7.6 Hz, H23), 2.71 (1H, m, H12), 2.52 (1H, m, H4), 6.88 (1H, d, *J*=7.6 Hz, H14), 2.39 (3H, s, H20), 2.05 (1H, m, H11), 1.50 (3H, s, H22), 1.43 (3H, s, H21). $^{13}$C NMR δ 193.0 (C6), 182.3 (C19), 177.1 (C2), 174.7 (C8), 142.1 (C17), 135.2 (C13), 127.4 (C15), 126.1 (C18), 119.8 (C14), 105.6 (C16), 104.6 (C7), 70.7 (C5), 61.3 (C10), 54.6 (C11), 42.1 (C3), 37.0 (C4), 26.9 (C12), 26.0 (C23), 25.8 (C22), 24.3 (C21), 18.7 (C20).

Reference NMR: (Tsuda et al., 2003)

**Selective extraction of sclerotia metabolites**

The selective extraction of sclerotia from *A. flavus* NRRL3357 (IBT3696), (IBT15934), NRRL 13462; *A. parvisclerotigenus* IBT16807 and *A. minislcerotigenus* IBT13353 was made from harvested sclerotia of a 7 day old cultivation on WATM and CYA agar (25°C in dark). The sclerotia were washed several times with Milli-Q (Millipore, Millford, USA) 0.22µm $H_2O$ and dried. The sclerotia were transferred to a 2 mL Eppendorf tube together with three stainsless steel balls (2*1mm and 1*5mm) and freezed with liquid N2 before mechanical crushed. The pulverized sclerotia was suspended in 1mL methanol and transferred to a 2mL vial with 1mL of 1:2:3 methanol:dichloromethane:ethylacetate and left for evaporation over night in a fume hood. The dried extract was resolved in 1mL methanol in ultrasonicated for 10min and then filtered with a 0.45µm PTFE filter to a clean vial for analysis.

**Data analysis of DIMS data**

The data analysis part comprises of two overall steps:

1) Pre-processing: Conversion of each raw continuum datafile into a single centroid mass spectrum, and the organization of the centroid spectra in a database/table.
2) Post-processing: Overall data analyses and mining.

The pre-processing step combines a sequence of raw scans in the raw data into one spectrum, followed by filtering and centroid estimation. The post-processing step contains the steps of binning the (continuum) centroid data into a binned grid, and the final data analysis. A detailed description of the method and tools can be found in (Hansen and Smedsgaard, 2007).

**Correspondence analysis**

NTSYS v2.21c (Exeter Software, Setauket, NY) was used for the analysis of binary data. Correspondence analysis was made on true data, consisting of binary data for secondary metabolites and sclerotia production. The first two CA axes explained 34% of the variation in the data.

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# 8 Discussion and conclusions

The introductive chapter of this thesis serves as a general overview of the current status on metabolomics for the most important *Aspergillus* species, selected for the first full genome sequencing. These genome sequences holds massive amounts of data, but the evolution of systems biology and bioinformatics promises fast annotations of possible genes.

The gap between the understanding of these putative annotated genes and the actual products is still large (Turner, 2010). *A. flavus* is one of the most investigated species due to its production of the extremely toxic aflatoxins and other mycotoxins, but only a fraction of the predicted genes for secondary metabolites have been mapped to the genome. Thus before addressing systematic metabolome research within this field, in-depth assessing of all potential chemistry from the questioned species must be fronted (Rank et al., 2010). A combinatorial approach is important to map the chemistry from two of the most important species within the genus *Aspergillus*: *A. fumigatus* and *A. flavus*/*A. oryzae* as well as to explore how the phenotype indirectly could aid the understanding of genomic data.

*A. fumigatus* is the most important pathogenic organism known to be involved in aspergillosis. The number of metabolites produced from this species is impressive, but the full understanding of the influence of these compounds on pathogenicity is lacking. The chemistry of this species is surveyed in the article "Metabolomics of *Aspergillus fumigatus*" (II). The accumulated knowledge of the potential chemistry is important for the evaluation of potential mycotoxigenic effects as well as for chemotaxonomy and the correlation of the number of predicted genes to the metabolites reported. As seen for the related, but yet different species *A. novofumigatus*, much of the chemical potential is comparable, however few of the compounds are identical. In the papers "*epi*-Aszonalenins A, B and C from *Aspergillus novofumigatus*" (III) and "Novofumigatonin, a New Orthoester Meroterpenoid from *Aspergillus novofumigatus*" (IV), four new metabolites are presented, which were not found elsewhere in *Aspergillus* section *Fumigati* at the time of publication. Novofumigatonin has some structural relations to fumigatonin, a metabolite reported from a marine-derived *A. fumigatus* (Okuyama et al., 1984), but never reported since. The *epi*-aszonalenins are related to the aszolanenins, which have been isolated from *Aspegillus zonatus*, a species formerly associated with section *Flavi*, but recently excluded with no new affiliation (Rigó et al., 2002). The discovery of the *epi*-aszonalenin class of benzodiazepines in the *Fumigati* has since the paper (III) been accompanied by discovery of aszonalenins in *A. fumigatus* (Hayashi et al., 2007) and the biosynthesis of acetylaszonalenin has been investigated in the teleomorph (sexual state) of *A. fumigatus*: *Neosartorya fischeri* (Yin et al., 2009). These later papers prove how important the discovery of novel compound classes within a chemotaxonomic related group of fungi is for the rest of the taxon and it

calls for a search of similar compounds throughout the group and particular within the clinical important species *A. lentulus* closely related to *A. fumigatus*. Benzodiazepines are known for their psychoactive properties and paradoxically these could theoretically be lead candidates. However, the specific bioactivity of these compounds remains to be determined and a nematicidal bioassay against *Caenorhabditis elegans* gave negative results (Hayashi et al., 2007).

Summarized, this work emphasizes the importance of unique chemistry for taxonomy and how closely related species have diverse biosynthetic features that make them unique.

The manuscript "Distribution of sterigmatocystin in filamentous fungi" (V) reviews the collection of taxonomically distant fungi all capable of production of the same, very important and potent mycotoxin: sterigmatocystin, which is a precursor in the biosynthesis of the extremely carcinogenic aflatoxins. The many years of research within this area and particularly problems with misidentification of species have resulted in some confusion that was questioned using HPLC-UV/Vis-DAD, LC-MS and LC-MS/MS. Despite that knowledge of sterigmatocystin goes back to the middle of last century and it is part of the most investigated class of mycotoxins, the list of sterigmatocystin producing genera is growing. With the expanding list of producers and evermore fungal strains being genome sequenced, the evolutionary investigations and implications for fungal secondary metabolite genes will hopefully deliver better explanations to the origins and broad distribution of the complex gene cluster.

This study found one new sterigmatocystin producer: *Penicillium inflatum*, potentially an unrecognized *Aspergillus* sp. as no evidence for sterigmatocystin production in *Penicillium* has ever been confirmed and phylogenic analysis also indicates close relations to other aspergilli. In addition, one known sterigmatocystin producer is upgraded to being an aflatoxin producer: *Aspergillus togoensis* (formerly *Stilbothamnium togoense*), which emphasizes the importance of modern analytical techniques and use of the One Species Many Compounds (OSMAC) methodology (Bode et al., 2002).

In chapter 6 and 7 some of the thoughts discussed in chapter 1 are evaluated. The important genome sequenced strain *A. oryzae* RIB40 is chemically analyzed and compared to the genome sequenced *A. flavus*. There is surprisingly little chemical coherence between the profiles, when grown under the same conditions, despite the fact that they have an estimated 99.5% homology at the genome level (Rokas et al., 2007). The isolation of the tremorgenic aflatrem precursor 13-desoxypaxilline and two precursors for parasiticolide A proves how different transcription of the same biosynthetic pathways can shift the overall chemical profile of individual isolates. The aflatrem gene cluster was annotated and analyzed recently in RIB40 and it was concluded that the gene cluster had a high homology with

the active *A. flavus*-aflatrem cluster (Nicholson et al., 2009). However, on the basis of no observed expression of the aflatrem gene cluster under normal growth conditions and a frameshift mutation in one of the latter biosynthetic genes, the authors concluded that the RIB40 aflatrem gene cluster was defective. Using a less biased approach than searching only for end-products and relying solely on other reports of no aflatrem production, we could isolate and prove 13-desoxypaxilline as a genuine RIB40 product. This demonstrates the advantages of a chemical confirmation for molecular genetics assessments, as this product fits the genetic indications of a frameshift mutation in the gene coding for AtmQ, which converts 13-desoxypaxilline to paspalicine/paspalinine (proposed immediate precursors for aflatrem). Two parasiticolide A analogues were also isolated and structure elucidated from RIB40. Parasiticolide A could be traced in small quantities using LC-MS/MS. The genes for parasiticolide A remains unmapped. A series of tentatively identified aflavinines were also discovered. Both the aflavinines and 13-desoxypaxilline (and aflatrem) are found in increased levels in the survival structures sclerotia, which the RIB40 strain is able to produce. To our knowledge there are only two known *A. oryzae* isolates with this capability. Despite the apparent chemical *in*homology, the RIB40 strain is presumably able to activate most of the secondary metabolic geneclusters of *A. flavus*, save the very well investigated aflatoxin pathway. The many disrupted biosynthetic pathways and the low frequency of sclerotia production in general, fits with the domestication theory for *A. oryzae*.

As the RIB40 strain is slowly mapped chemically, many important conclusions can be drawn to the genome, as discussed above. Possible points of mutation in incomplete pathways can be suggested to bioinformatics as well as a better understanding of differences in overall regulation and genuine metabolomics can be investigated and coupled to transcriptomics.

In chapter 7 a different approach to mapping the chemical potential of a species is assessed. A substantial number of isolates (382) of *A. flavus*, *A. oryzae* and close relatives in *Aspergillus* section *Flavi* were analyzed with different methods to document the chemical diversity in these species and search for potential new chemical markers. Chemically the Direct Inject Mass Spectrometry (DIMS) method was assessed as a quick method for separating the broad group of large sclerotium producing *A. flavus* isolates from the more potent small sclerotium producers, such as *A. minisclerotigenes* and *A. parvisclerotigenes* and other aflatoxin G producers. The method has previously proved successful in clustering different species of *Penicillium* (Smedsgaard and Frisvad, 1997; Smedsgaard, 1997; Hansen and Smedsgaard, 2007). The results were ambiguous to some extent, displaying tendencies that correlated with the known species, but the chemistry was presumably too alike, for the very large number of strains investigated, to allow for a clear clustering of the species with DIMS. One of the shortcomings of this method, ion suppression, plays an important role in the lost information and lower scoring. Careful analysis of the data , however, made

is possible to pinpoint important chemical markers for the clustering and thereby also for the chemotaxonomy of the species: the CPA analogue speradine A proved an important ion, which was confirmed with manual de-replication. A correspondence analysis of only *A. flavus* and *A. oryzae* isolates based on a set of unique markers proved the vague results from the DIMS study: namely that *A. flavus* and *A. oryzae* are difficult to segregate chemically, though unique metabolic features are present. This reflects the knowledge from the genome sequenced strains and general molecular genetic studies, which have tried to describe the very broad biodiversity hosted by *A. flavus*. Chemically, *A. flavus* is a very diverse species, but a core set of metabolites are almost always present; aflatoxin, kojic acid, flavimine, aflavinines, aflatrem, aspergillic acid and FNI. There exists a group of special, yet unidentified metabolites, which are predominant in very few isolates. There also appears to be unique metabolites to *A. oryzae*, though they logically should also be possible to find in isolates of *A. flavus*, since this is the wild type species. The correlation of these metabolites to the two predicted diverging NRPS genes will be particularly interesting to establish, if these really are unique features (Rokas et al., 2007).

The impact of analyzing enough isolates to ensure that the entire chemodiversity is covered enables us to select possible candidates for genome sequencing based on the phenotypic expression on a few media. Though the sequenced isolates of *A. flavus* (NRRL3357) and *A. oryzae* (RIB40) apparently are capable of expressing most of the perceived chemodiversity within the group, some compounds may prove impossible to express, even with the advantages of using OSMAC methodology or epigenetic modifiers, because of genetic mutations. The type of *A. flavus* (NRRL 1957) was for example not targeted for the first genome sequencing, as it is non-aflatoxigenic.

Overall a series of methodologies and approaches are questioned in this thesis and these initial investigations prove an untapped potential for use of the chemical phenotype to describe genomic features.

## Perspectives

The combinatory use of different methods for assessing the chemodiversity and targeting important novel compounds should be used in a campaign to fully map the key species of *Aspergillus*. The effort needed to do so can, however, not be underestimated and the use of new UPLC systems with accompanying UHR-MS instruments in combination with automated data scavenging methods will be necessary for mapping of all metabolites. These in-depth studies should have offset in the isolates found here to have an extreme chemical profile, such that unique metabolites can be isolated in amounts that allow for structure elucidation.

The usages of OSMAC and epigenetic modifiers have proved advantageous to squeeze cryptic metabolites out of the genomes of other species. I have conducted some preliminary tests with *A. flavus* and *A. oryzae* on complex media with added epigenetic modifiers, and these results were promising in terms of the potential of this approach. However, for a controlled study minimal media should be used before combining with more complex substrates in order to evaluate the effect of each parameter independently.

The potential chemical markers for *A. flavus*, like: flavimine, GYLE, FLIT and FUT, and for *A. oryzae*: NO2 and NB, are extremely important, since they apparently are the metabolites that differentiates these two closely related species the most. Some of these were targeted for isolation and structure elucidation during this work, but the attempts were however not successful and prompts for a large extraction than was done in this work. Several other compounds in *A. oryzae* RIB40 were noted to be very interesting. Establishment of the structures of these metaboliteswill hopefully further contribute to the phenotypic linking of *A. flavus* and *A. oryzae* and be an aid for the understanding of genomic and transcriptional differences between these related species.

Altogether, a better integration of bioinformatics, molecular genetics and chemistry will be paramount for advances in the understanding of both the phenotypes as well as mapping of the secondary metabolite gene clusters. A focused collaboration on mapping some of these apparently important metabolites to the genome through deletion and overexpression of secondary metabolite pathway gene clusters, will be of great impact to the *Aspergillus* community.

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# Appendix A

**Isolates used for DIMS study (X for isolates also used in correspondence analysis)**

| CA | Triplicate | IBT no. | Species | Comment | Sclerotia | Original No. |
|---|---|---|---|---|---|---|
| | | 3595 | oryzae | | 0 | NRRL 1988 |
| X | | 3599 | flavus | | 0 | CBS 102.22 |
| X | D | 3602 | flavus | | 0 | NRRL 484 |
| X | | 3602 | flavus | | 0 | NRRL 484 |
| | T | 3604 | toxicarius | | S | CBS 561.82 |
| X | D | 3605 | flavus | Type | L | NRRL 1957 |
| X | D | 3606 | flavus | | 0 | AMAZ 14 |
| X | | 3610 | flavus | Type | L | NRRL 1957 |
| X | | 3618 | flavus | | M(L) | NRRL 3251 |
| | D | 3620 | flavus | | L | AMAZ 20 |
| X | | 3624 | flavus | | L | HB 1 |
| X | | 3629 | flavus | | 0 | GR o11 |
| | | 3630 | oryzae | | 0 | ATCC 22787 |
| | | 3631 | oryzae | | L | NRRL 482 |
| | T | 3635 | flavus | | L | JSCN 17 |
| X | | 3638 | flavus | | 0 | NRRL 453 |
| | | 3639 | flavus | | 0 | ATCC 44564 |
| | T | 3640 | columnaris | | L | NRRL 5821 |
| | | 3641 | terricola | | 0 | CBS 485.65 |
| X | | 3642 | flavus | | L | CBS 569.65 |
| | | 3646 | flavus | | L | HA 1 |
| X | | 3649 | kambarensis | Type | 0 | CBS 542.69 |
| | T | 3651 | flavus | * | 0 | CBS 121.62 |
| | T | 3657 | columnaris | Type | 0 | CBS 485.65 |
| | | 3660 | columnaris | | 0 | CBS 242.65 |
| | | 3663 | flavus | | L | BB1 |
| | | 3667 | flavus | | L | AMAZ 19 |
| X | | 3668 | flavus | | 0 | CBS 103.13 |
| | D | 3671 | flavus | | 0 | ATCC 32591 |
| X | | 3688 | oryzae | | 0 | NRRL 695 |
| X | | 3696 | flavus | FGS | L | NRRL 3357 |
| | | 3697 | flavus | | L | MON 4 |
| | | 3701 | flavus | | 0 | ATCC 44564 |
| X | | 3706 | flavus | | L | CBS 569.65 |
| X | | 3810 | flavus | | 0 | NRRL 3502 |
| X | | 3811 | flavus | | 0 | NRRL 6270 |
| X | | 3819 | flavus | | 0 | CBS 110.55 |
| | | 3820 | flavus | | L | NRRL 5565 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| X | | 3825 | flavus | | 0 | NRRL 6551 | |
| X | | 3834 | oryzae | | 0 | NRRL 449 | |
| X | | 3835 | oryzae | | 0 | NRRL 5593 | |
| X | | 3837 | flavus | | L | NRRL 6556 | |
| X | | 3839 | oryzae | | 0 | NRRL 3485 | |
| | | 3840 | minisclerotigenes | | S | NRRL 6444 | |
| | | 3850 | parvisclerotigenus | | S | NRRL A-11612 | |
| | | 4137 | flavus | | L | MOUS S277 | |
| | | 4157 | flavus | | L | MOUS S276a | |
| | | 4161 | flavus | | L | MOUS S7 | |
| | | 4162 | flavus | | L | MOUS S298 | |
| X | | 4192 | flavus | | 0 | CBS 119.62 | |
| X | | 4193 | flavus | | 0 | CBS 108.30 | |
| X | | 4194 | flavus | | 0 | CBS 118.62 | |
| X | | 4195 | flavus | | L | CBS 130.61 | |
| | | 4196 | flavus | | L | CBS 131.61 | |
| X | | 4197 | flavus | | L | CBS 247.65 | |
| X | | 4198 | flavus | | L | CBS 120.62 | |
| | | 4199 | flavus | | 0 | CBS 107.45 | |
| X | | 4366 | oryzae | | 0 | NRRL 2220 | |
| X | | 4371 | oryzae | | 0 | NRRL 451 | |
| | | 4377 | toxicarius | Type | 0 | CBS 822.72 | |
| | T | 4387 | parasiticus | | 0 | CBS 260.67 | |
| X | T | 4388 | flavus | | L | CBS 569.65 | |
| | T | 4401 | flavus | | 0 | ATCC 32591 | |
| X | | 4402 | flavus | Type | L | CBS 501.65 | |
| | | 4514 | flavus | * | 0 | SNACC 7A | |
| X | | 4601 | oryzae | | 0 | NRRL 1911 | |
| X | T | 5573 | flavus | | 0 | FRR 2874 | |
| | | 5693 | flavus | | L | FN 1 | |
| | T | 5694 | flavus | | L | AHYA 10 | |
| | | 5695 | flavus | | L | JSCN 9 | |
| | | 5697 | flavus | | L | FIJ 3 16 | |
| | | 5698 | flavus | | 0 | AR 1 1 | |
| | | 5750 | flavus | | L | IMI 246656 | |
| | | 5777 | flavus | | L | Jordnød-muse | |
| X | | 5778 | flavus | Type | L | NRRL 1957 | |
| X | | 5798 | flavus | | L | ETH 2-51 | |
| | | 5799 | flavus | | L | HVN 2 | |
| | | 5800 | flavus | * | 0 | 100-T | |
| | | 5801 | flavus | | L | SLBO 880 7350B | |
| | T | 5802 | flavus | | L | | 16 |
| | | 6069 | flavus | | L | AMAZ 15 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | 6230 | flavus | * | 0 | 419-2 |
| X | | 6462 | flavus | | 0 | FRR 2874 |
| | | 6463 | flavus | | L | Muggen figen 1 |
| | | 6743 | flavus | | 0 | aflatoxin 12 |
| | | 6744 | flavus | | 0 | 15 (svag aflatoxin) |
| | | 6745 | flavus | | 0 | Gul fluo. 13 |
| | | 6746 | flavus | * | 0 | Gul fluo. 1 |
| | T | 10963 | minisclerotigenes | | S | SRRC 2105 |
| | | 11107 | flavus | | L | IBT 10245 |
| | | 11108 | flavus | | L | IBT 10246 |
| | | 11193 | flavus | * | 0 | 8A 2-59 |
| | | 11194 | flavus | | L | 8B 1-78 |
| | | 11450 | flavus | | L | AJL 9179-6a |
| X | | 11586 | flavus | | 0 | CBS 110.27 MA2 |
| X | | 11587 | flavus | | 0 | CBS 108.24 MA2 |
| | | 11588 | nomius | | 0 | CBS 131.54 |
| | | 11589 | flavus | | L | DES-N |
| X | D | 11590 | flavus | | 0 | CBS 542.69 MA2 |
| X | | 11594 | flavus | | L | CBS 116.48 |
| X | | 11597 | flavus | | 0 | CBS 112.45 MA2 |
| X | | 11763 | flavus | | 0 | CBS 108.45 |
| X | | 11764 | flavus | | L | CBS 110.45 |
| X | | 11793 | flavus | | L | CBS 113.49 |
| X | | 11846 | oryzae | | 0 | CBS 115.33 |
| X | T | 11863 | flavus | | L | CBS 115.37 |
| | | 11888 | flavus | | 0 | 441-A 5 |
| | T | 11892 | flavus | | L | 30665-Z |
| | | 11898 | flavus | | L | 433-31365 |
| | | 11899 | flavus | | L | 441-A1 |
| | | 11900 | flavus | | 0 | 441-A3 |
| | | 11901 | flavus | | 0 | 30702-A2 |
| | | 11902 | flavus | | 0 | 31029-C2 |
| | | 11903 | flavus | | L | 31179-F1 |
| | | 11904 | flavus | | L | 31292-7 |
| | | 11912 | flavus | | 0 | 441-P2 |
| | | 11917 | flavus | | 0 | TOVE-N |
| | | 12081 | flavus | | L | Foder-contrymix 1 |
| | | 12120 | flavus | * | L | 31139 F |
| | | 12225 | tamarii | | S | OB 2 No.8 |
| | | 12282 | flavus | | 0 | OD 3 No.1 |
| | | 12290 | flavus | * | 0 | OD 5 No.1 |
| | D | 12613 | flavus | | L | PV a7 |
| | | 12614 | flavus | | L | KVL 82 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | 12615 | flavus | | L | PV a4 grøn |
| | | 12618 | flavus | | L | PV 8 |
| | D | 12652 | flavus | | L | SEED-7 |
| X | | 12653 | flavus | | 0 | # 49 |
| X | | 12655 | flavus | | L | # 139 |
| | | 12656 | flavus | | 0 | SEED-9 |
| | D | 12657 | flavus | * | 0 | SEED-11 |
| X | | 12658 | flavus | | 0 | # 38 |
| X | | 13083 | flavus | | 0 | ATCC 44054 Hvid |
| | | 13084 | columnaris | | 0 | ATCC 44310 |
| X | | 13097 | flavus | | L | ATCC 26850 |
| | T | 13333 | minisclerotigenes | | S | C1 SCL !!! |
| | | 13334 | flavus | | L | C 92-9 |
| | | 13335 | minisclerotigenes | | S | C4 |
| | | 13353 | minisclerotigenes | | S | M9 ISO A6 SCL!!! |
| | | 13414 | flavus | | L | E5-DR-4 |
| X | | 13446 | flavus | | L | Byg 20-92 |
| | | 13458 | flavus | | 0 | Havre 17-92 |
| | | 13459 | flavus | | L | Byg 7-92 |
| | | 13762 | flavus | | L | 2E 8-13 |
| | | 13809 | flavus | | L | 2E 9-8 |
| | | 13810 | flavus | | L | 2E 9-7 |
| X | T | 13864 | flavus | * | 0 | NRRL 490 |
| | | 13964 | flavus | | 0 | ALK 21 |
| | | 14028 | flavus | | 0 | ALK 22 |
| | | 14154 | flavus | | L | Syd 5 |
| | | 14201 | minisclerotigenes | | S | IBT 6809 |
| | | 14374 | flavus | | L | U-Sp-E8a |
| | T | 14534 | oryzae | | 0 | A1568-B 1 |
| | | 14575 | oryzae | | 0 | #Closed?# |
| | | 14647 | zhaoqingensis | Type | 0 | CBS 399.93 |
| | | 14870 | flavus | | 0 | 3A 3-3 |
| | | 15016 | flavus | | L | 6A-1 |
| | | 15017 | flavus | | 0 | 3D-1 |
| | | 15018 | flavus | | L | 21A-3 |
| | | 15021 | flavus | | L | 18C-1 |
| | | 15164 | flavus | | L | GC 79-2+K |
| | | 15312 | flavus | * | 0 | C94-1-4 |
| | | 15443 | flavus | | L | HvA 2 |
| | | 15591 | flavus | | L | King 3-139-2 |
| | | 15606 | flavus | | L | WAR 4 |
| | | 15613 | flavus | | L | Rah-FNCC 6107 |
| | D | 15614 | flavus | * | 0 | Rah-FNCC 6109 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | 15652 | flavus | | L | FRR 4288 |
| | T | 15674 | flavus | | 0 | MF 94-1 |
| | | 15698 | flavus | | 0 | 1021-2-bund (H) |
| | | 15705 | flavus | | L | RQ 94-4 ACMBO 287 Sep.19/92 |
| | | 15706 | flavus | * | 0 | LB 94-1 |
| | | 15711 | flavus | | L | 12A 2-5 |
| | | 15712 | flavus | | 0 | 18B-1 |
| | T | 15713 | flavus | | L | 9B-2 |
| | | 15714 | flavus | | L | 10G1-5 |
| | | 15837 | flavus | | L | DAOM 174568 |
| | | 15915 | flavus | | 0 | 31 R-3 No.1 |
| | | 15934 | flavus | | L | J7 |
| | | 15959 | flavus | | L | VK-7 No.3 |
| X | T | 16338 | flavus | | L | 17/A2/RMF 9505 |
| X | | 16343 | flavus | | 0 | 25/F1/RMF 9513 |
| | | 16350 | flavus | | 0 | 35/E3/RMF 9522 |
| | | 16412 | flavus | * | L | RMF 7133 |
| X | | 16414 | flavus | | L | RMF 7621 |
| X | | 16415 | flavus | | 0 | RMF 7828 |
| X | | 16416 | flavus | | L | RMF 7830 |
| | | 16417 | flavus | * | 0 | RMF 7988 |
| X | | 16424 | flavus | | 0 | RMF 7673 |
| X | | 16760 | flavus | | 0 | DM G789 |
| | | 16807 | parvisclerotigenus | | S | N95-15-6 |
| | | 16808 | parvisclerotigenus | | S | N95-15-5 |
| X | | 17644 | oryzae | | 0 | Anne-4 |
| X | | 17706 | oryzae | | 0 | CF 1.1 |
| X | | 17707 | oryzae | | 0 | A 1560 |
| | | 18106 | flavus | * | 0 | Sadel 4-95 |
| | | 18119 | flavus | | 0 | 18-13-2C ys2 |
| | | 18438 | flavus | | L | VESTB 7G |
| | | 18439 | flavus | | L | VESTL 9G |
| | T | 18648 | flavus | | 0 | DANT 38AB 11/1-96 |
| | | 18735 | oryzae | | 0 | CF 2.1a W |
| | | 18932 | flavus | *** | 0 | DANL 47A 11/1-96 |
| | | 19411 | flavus | * | 0 | A-4 |
| | | 19412 | flavus | * | 0 | Silo 32-5 |
| | | 19413 | flavus | * | 0 | B-2 |
| | | 19749 | flavus | | L | (5) |
| X | | 19750 | flavus | | L | (2A) |
| | | 19902 | flavus | | 0 | KELK 11K2 |
| | | 19903 | flavus | | 0 | KELK 35K6 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | 19904 | flavus | * | 0 | KELK 33K1 |
| X | | 20313 | flavus | | L | RN 4-3 |
| | | 20577 | flavus | | L | LP 25 |
| | | 20608 | flavus | * | 0 | BI 9609250066 (b) |
| X | | 20945 | flavus | | 0 | Ven 97 M 25B-1 |
| | | 20989 | tamarii | | 0 | Ven 97 M 4B-11B |
| | | 21010 | flavus | * | M | Ven 97 M 104-2B |
| | | 21066 | flavus | * | 0 | Ven 97 T 6-4 |
| X | | 21067 | flavus | | L | Ven 97 M 67B-9 |
| X | | 21068 | flavus | | L | Ven 97 M 55-13 |
| X | | 21069 | flavus | | L | Ven 97 M 23B-12 |
| X | T | 21070 | flavus | | L | Ven 97 M 67B-14 |
| | | 21090 | pseudotamarii | | 0 | NRRL 25518 |
| | | 21091 | caelatus | Type | 0 | NRRL 25528 |
| | | 21092 | pseudotamarii | Type | L | NRRL 25517 |
| | | 21093 | pseudotamarii | | 0 | NRRL 25519 |
| X | | 21140 | flavus | | 0 | CCRC 32145 |
| | | 21141 | flavus | | L | CCRC 32422 |
| X | | 21142 | flavus | | 0 | CCRC 32140 |
| X | | 21145 | flavus | | 0 | CCRC 32148 |
| | | 21217 | minisclerotigenes | | S | ESC 2A |
| | | 21278 | flavus | | L | Industrial |
| X | | 21279 | flavus | | L | HCF 6530 |
| X | | 21280 | flavus | | L | Industrial |
| X | | 21283 | flavus | | 0 | HFC 6539 |
| | | 21284 | flavus | | L | Industrial |
| X | | 21285 | flavus | | L | HCF 6601 |
| | | 21315 | flavus | * | 0 | HB3 |
| | | 21423 | flavus | * | L | 11-VEST-RÅVARER |
| | | 21427 | flavus | | L | CBS 131.61 |
| X | | 21429 | oryzae | | 0 | CBS 201.75 |
| X | | 21430 | flavus | | L | CBS 625.66 |
| X | T | 21431 | flavus | | 0 | CBS 616.94 |
| | | 21432 | flavus | * | 0 | CBS 109.31 |
| | | 21433 | flavus | * | 0 | CBS 107.45 |
| | T | 21434 | flavus | | 0 | CBS 113.32 |
| X | | 21435 | flavus | | L | CBS 816.96 |
| | | 21436 | flavus | | 0 | CBS 103.57 |
| | | 21437 | flavus | | L | CBS 130.61 |
| X | | 21438 | oryzae | | 0 | CBS 818.72 |
| X | | 21439 | oryzae | | 0 | CBS 125.59 |
| X | | 21440 | oryzae | | 0 | CBS 205.89 |
| X | | 21442 | flavus | | L | CBS 119.62 |

| | | | | | | |
|---|---|---|---|---|---|---|
| X | | 21443 | flavus | | 0 | CBS 118.62 |
| X | | 21444 | flavus | | 0 | CBS 117.62 |
| X | | 21445 | oryzae | | 0 | CBS 673.92 |
| X | | 21446 | oryzae | | 0 | CBS 672.92 |
| X | | 21447 | flavus | | L | CBS 573.65 |
| X | | 21448 | flavus | | 0 | CBS 247.65 |
| X | | 21449 | flavus | | L | CBS 282.95 |
| X | | 21451 | oryzae | | 0 | CBS 110.47 |
| X | | 21452 | oryzae | | 0 | CBS 110.47 |
| X | | 21455 | flavus | | 0 | CBS 108.30 |
| X | | 21456 | oryzae | | 0 | CBS 674.92 |
| X | | 21457 | flavus | | L | CBS 109.45 |
| X | | 21458 | oryzae | | 0 | CBS 570.65 |
| | | 21556 | flavus | | L | Kasavamel 203-12 |
| | | 21557 | flavus | | L | Kasavamel 203-7 |
| | | 21642 | sojae | | 0 | CBS 100928 |
| X | | 21707 | flavus | | 0 | ITTA A1 |
| | | 21862 | flavus | * | 0 | K 2-1-99 |
| | | 21933 | arachidicola | | 0 | Lene lange |
| | | 21951 | flavus | * | 0 | IM4-3 |
| | | 22301 | flavus | | 0 | HT 445 |
| | T | 22566 | terricola | | 0 | WB 4680 |
| X | | 22573 | flavus | | 0 | ITEM FV 10 |
| X | | 23106 | flavus | | L | NGC 7226 |
| X | | 23418 | flavus | | L | CR 18-13 |
| X | | 23476 | flavus | | 0 | Wheat 10 |
| | | 23530 | nomius | | 0 | NRRL 25585 |
| X | | 23531 | flavus | | L | NRRL 6541 |
| X | | 23532 | flavus | | L | NRRL 29254 |
| X | T | 23533 | flavus | | L | NRRL 13462 |
| | | 23534 | nomius | | 0 | NRRL 5919 |
| | | 23535 | bombycis | | 0 | NRRL 25593 |
| | | 23536 | bombycis | Type | 0 | NRRL 26010 |
| | | 23537 | bombycis | | 0 | NRRL 29253 |
| X | | 23775 | flavus | | 0 | C 1a |
| | | 23830 | oryzae | | 0 | TI-4-915 |
| | | 23831 | oryzae | | 0 | TI-3-915 |
| X | | 23967 | flavus | | 0 | EXF 438 |
| | | 24042 | flavus | | 0 | IZ 29 |
| | | 24043 | flavus | | 0 | IZ 13 |
| | | 24044 | flavus | | 0 | IZ 78 |
| X | | 24626 | flavus | | L | Peber 2 |
| | | 24628 | minisclerotigenes | | S | Peber 11 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | 24629 | minisclerotigenes | | S | KFN 3-1 |
| | | 24827 | flavus | | 0 | Thepose 7 |
| | | 24828 | flavus | * | 0 | Thepose 5 |
| X | | 24829 | flavus | | 0 | Thepose 6 |
| | | 24872 | flavus | | 0 | MZKI A482 |
| | | 24985 | flavus | | 0 | KIR 39, 323.95 |
| | | 24995 | flavus | * | 0 | MZKI A330 |
| | | 24996 | flavus | | 0 | MZKI A304 |
| | | 24997 | flavus | | 0 | KIR 51 |
| X | | 25733 | flavus | | 0 | Kjop 22-1x |
| | | 25736 | nomius | | 0 | jop 670-9x |
| X | | 25739 | flavus | | L | L 2x |
| | T | 26276 | oryzae | | 0 | Industrial |
| | | 26469 | oryzae | | 0 | Industrial |
| | | 26470 | oryzae | | 0 | Industrial |
| | | 26548 | flavus | | 0 | Løgfrø 2 |
| X | | 26549 | flavus | | L | Tomatfrø1 |
| | | 26767 | flavus | | 0 | J-K 15 |
| | | 26768 | flavus | | 0 | J-K 16 |
| | | 26769 | flavus | | 0 | J-K 17 |
| | | 26793 | flavus | | L | J-K 41 |
| | | 26794 | flavus | | 0 | J-K 42 |
| | | 26795 | flavus | | L | J-K 43 |
| | | 26813 | flavus | | L | J-K 63 |
| | | 26922 | flavus | * | 0 | Capybara 1 |
| | | 27124 | flavus | * | 0 | M29N3 |
| | D | 27125 | arachidicola | | 0 | M8H1 |
| | | 27126 | XX | | 0 | M8H5 |
| | | 27127 | flavus | * | 0 | M13H3 |
| | T | 27128 | flavus | * | 0 | M7H3 |
| | | 27129 | arachidicola | | 0 | - |
| | | 27130 | flavus | * | 0 | - |
| | | 27131 | flavus | * | 0 | - |
| | | 27136 | arachidicola | | 0 | M8H5A |
| X | | 27137 | flavus | | 0 | M8H5B |
| | | 27177 | minisclerotigenes | | S | T4N33 |
| | | 27179 | flavus | | L | |
| X | | 27181 | flavus | | L | EH1 |
| | | 27182 | tamarii | | 0 | T3N21 |
| | | 27183 | arachidicola | | 0 | M8H4 |
| | | 27185 | arachidicola | | 0 | M7H4 |
| X | | 27187 | flavus | | L | M12N1 |
| X | | 27188 | flavus | | 0 | M34N2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | 27189 | flavus | | 0 | M29N3 |
| | | 27191 | caelatus | | 0 | M13H3 |
| X | T | 27192 | flavus | | 0 | LLF 5 |
| X | | 27193 | flavus | | L | M22 |
| | | 27195 | minisclerotigenes | | S | T40N1 |
| | | 27196 | minisclerotigenes | Type | S | M22N3 |
| | | 27197 | minisclerotigenes | | S | M32N4 |
| | | 27199 | minisclerotigenes | | S | LLF 39 |
| | | 27200 | minisclerotigenes | | S | LLF 37 |
| | | 27213 | minisclerotigenes | | S | M8H5A |
| | | 27214 | minisclerotigenes | | S | M8H2A |
| | | 27215 | arachidicola | | 0 | M7H3B |
| X | | 27216 | flavus | | 0 | M12H5B |
| | | 27218 | arachidicola | Type | 0 | M7H3A |
| X | | 27219 | flavus | | L | ESC 2B |
| | | 27221 | flavus | | 0 | M8H2B |
| | | 27222 | minisclerotigenes | | S | M8H2D |
| | | 27370 | flavus | * | 0 | J-K 354 |
| | | 27864 | arachidicola | | 0 | NRRL 3353 |
| | | 27925 | flavus | | L | KACC 41734 |
| | | 27926 | flavus | * | 0 | KACC 41730 |
| | | 27988 | flavus | | 0 | bel 1000-1 |
| | | 27989 | flavus | * | 0 | KACC 41730 B |
| X | T | 28103 | oryzae | FGS | L | RIB 40 |
| | | 28383 | flavus | | L | Lyngby Sø 6 |
| X | | 28413 | flavus | | L | Lyngby Sø 4 |
| | | 28414 | flavus | | L | Bagsværd sø 1 |
| X | | 28518 | flavus | FGS | 0 | NRRL 3357 WT |
| | | 28519 | flavus | | L | TJW 79.13 Multicopy lac A |
| | | 29301 | flavus | | L | |
| | | 29302 | flavus | | 0 | |
| | | 100018 | oryzae | | 0 | CF 1.1 Wildtype |
| | | 12193grøn | flavus | | 0 | AJL 1531/2-3 |
| | | 12193gul | flavus | | 0 | AJL 1531/2-3 |
| | | 5696grøn | flavus | | 0 | FIJ 7 |
| | | 5696hvid | flavus | * | 0 | FIJ 7 |
| | T | 6809grøn | minisclerotigenes | | S | - |
| | | 6809hvid | minisclerotigenes | | S | - |

# Appendix B

**Metabolites and markers used in correspondence analysis**

Kojic acid
Kojic acid dimer
Cyclopiazonic acid
Asperfuran
Aspirochlorine
Parasiticolide A
Parasiticolide B
NB
NO2
Citreoisocoumarin
Cyclopiamin
Flavimine
Ustilaginoidin
Indol
FLIT
GYLE
VERNO
FUT
PRS
Aflatoxin B1
Aspergillic acid
PAL
EPIF
Aflavinins
Aflatrems
Aflavarins
Versicolorins
FNI
Sclerotia

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